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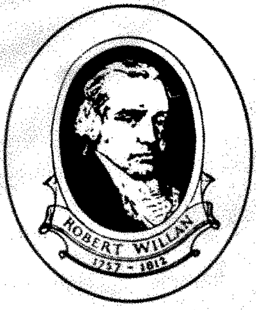

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Serum type III procollagen peptide, dynamic liver function tests and hepatic fibrosis in psoriatic patients receiving methotrexate

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SUMMARY

The serum level of the aminoterminal peptide of type III procollagen (P3NP) was measured in 51 psoriatic patients on long-term, once weekly, low-dose methotrexate and in a control group of patients with extensive psoriasis who had never had systemic treatment. Serum P3NP levels were normal in all control patients, but were elevated in the majority of methotrexate-treated patients, even those with normal or non-specific liver histology. Although the highest P3NP values were found in the groups of patients with fibrosis and cirrhosis, isolated P3NP measurements did not discriminate between individuals with and without significant liver pathology.

Neither standard nor dynamic liver function tests were able to identify patients with significant liver damage and in most cases results were in the normal range.

Methotrexate is a valuable drug in the treatment of severe psoriasis but the development of hepatic fibrosis in some patients may necessitate its withdrawal. Fibrosis and cirrhosis may develop without detectable changes in liver enzymes, and neither radionuclide nor ultrasound examination are sufficiently sensitive or specific to detect fibrosis reliably.[1] Liver biopsy is accepted as the definitive test for hepatic fibrosis, but is associated with a small but significant risk of morbidity and mortality. Since psoriatic patients may require repeated biopsies during the course of prolonged treatment with methotrexate, a serological test reflecting hepatic fibrosis would be of great value.

During liver fibrosis there is an increase in hepatic collagen, predominantly of types I and III. Collagen molecules are derived from procollagen by cleavage of terminal peptides which are

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released into serum. Recently a radioimmunoassay has been developed to measure the aminoterminal peptide of type III procollagen (P3NP).[2] Studies of fibrotic liver disease using this assay have demonstrated that serum P3NP is raised in patients with primary biliary cirrhosis and chronic active hepatitis but not in those with chronic persistent hepatitis.[3] During episodes of hepatitis in patients with alcoholic cirrhosis, serum P3NP levels are markedly elevated and then gradually decrease as the acute episode resolves.[4]

Since development of hepatic fibrosis and cirrhosis eventually reduces functional capacity by disruption of blood flow and replacement of hepatocytes by collagen, measurement of hepatic functional capacity may also reflect the development of fibrosis. The galactose elimination capacity (GEC) is dependent upon hepatic functional cell mass.[5] The bromosulphophthalein (BSP) excretion test[6] measures partial hepatic functions: BSP-k_i measures overall excretory function including blood flow; BSP-k_1, hepatic blood flow and uptake; and BSP-k_2, biliary excretory capacity.

This study examined a group of psoriatic patients receiving long-term methotrexate therapy to determine whether their serum P3NP levels or dynamic liver function tests were abnormal. We examined the relationship between serum P3NP concentration, liver function, and liver histology and tried to establish whether either serum P3NP levels or dynamic liver function tests could reliably indicate the presence of hepatic damage and thus provide an alternative to liver biopsy for regular screening of these patients.

METHODS

Patients

The study group consisted of 51 patients with severe psoriasis (age range 22–69 years, median 47 years) who had received oral methotrexate once weekly in cumulative doses of between 545 and 10024 mg (median 2538 mg) for between 1 and 13 years (median 5·2 years). All tests were performed during a 48-h period in hospital.

Serum was collected for P3NP determination at least 5 days after the patient's last methotrexate dose and immediately prior to liver biopsy. All samples were stored at −20°C until analysis at the end of the study using a standard radioimmunoassay (RIA-gnost® Prokollagen III peptid—Behringwerke AG, Marburg, FRG; intra-assay CV = 8·5%: inter-assay CV ($n = 10$) = 8·5%).

For both BSP and GEC tests, values below the lower limit of the normal range are regarded as indicative of liver damage. BSP excretion kinetics were measured in patients who did not give a history of atopy or adverse drug reactions by the method of Hacki[6] using a dose of 5 mg/kg body weight. The lower limit of the normal range (mean − 2SD) was: BSP-k_i = 9·4%/min; BSP-k_1 = 11·3%/min; BSP-k_2 = 1·5%/min. GEC was determined by the method of Tygstrup *et al.*,[5] using a standard dose of galactose of 500 mg/kg body weight and sampling over a 70-min period (lower limit of normal range = 4·7 mg/min per kg).

Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase (GGTP) and bilirubin were measured by standard autoanalyser techniques.

Liver biopsies were obtained by the Menghini technique and immediately fixed in 10% formaldehyde solution. Sections of these biopsies were stained using: haematoxylin and eosin; Perls' stain for iron; PAS before and after diastase; orcein for elastic tissue, hepatitis B surface-antigen and metallothionein; and untoned reticulin and haematoxylin/picrosirius red for collagen. All the biopsies were assessed semi-quantitatively, without knowledge of methotrexate dosage, by one observer (N.Y.H.) and allocated to one of five groups according to severity of liver damage: (i) normal histology, (ii) steatosis (fatty change) alone, (iii) inflammation (± steatosis) without fibrosis, (iv) fibrosis (± steatosis ± inflammation) and (v) cirrhosis.

Control groups

In our study, serum P3NP levels were examined in two control groups: (i) 18 healthy patients presenting with non-inflammatory skin disorders e.g. warts or basal cell carcinomata (normal group; age range 20–73 years, median 40 years) and (ii) 18 patients with moderate to severe active psoriasis who had never received systemic treatment for psoriasis (psoriatic controls; age range 20–76 years, median 33 years). All control patients had normal liver function tests and no history or clinical features indicative of liver disease.

Statistical methods

The non-parametric statistical methods used in this paper were applied to the data using the computer programs found in the Statistical Package for the Social Sciences (S.P.S.S.) All probability values quoted are for a two-tailed level of significance.

RESULTS

Histology

Twenty (39%) patients had a liver histology that was described as normal (13) or that showed steatosis alone (7); 18 patients (35%) had inflammation without fibrosis; and 13 patients (25% of the whole group) had evidence of fibrosis (10) or cirrhosis (3).

Procollagen peptide levels

The normal reference range for serum levels of P3NP quoted by the assay manufacturers is 4–12 ng/ml. None of the subjects in the normal or psoriatic control groups had elevated serum P3NP levels (range 2·0–11·8 ng/ml) (Fig. 1), and there was no difference in P3NP values between the

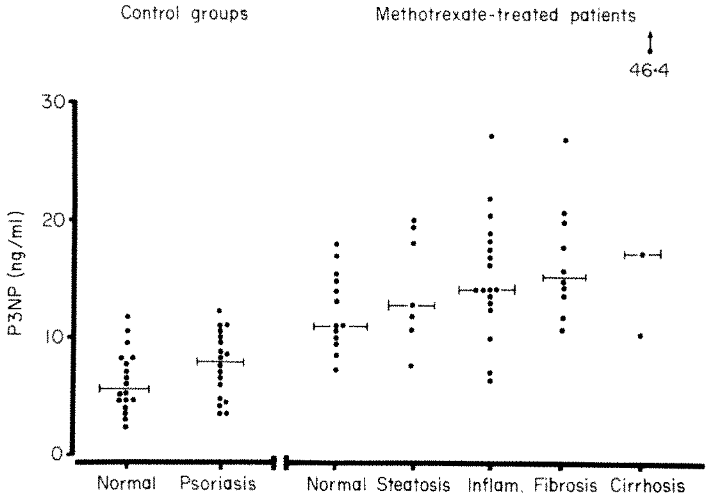
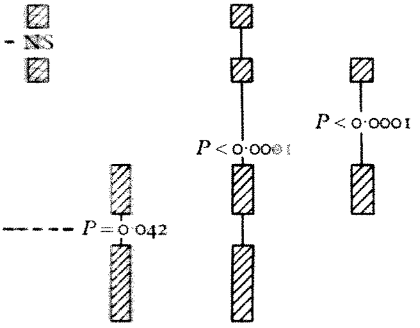


FIGURE 1. Serum P3NP levels in methotrexate-treated psoriatics divided according to histological status and in control groups. Medians are indicated by bars. The normal range for P3NP is 4–12 ng/ml.

TABLE 1. Serum P3NP concentrations in controls and in methotrexate-treated psoriatics grouped according to liver histology. Hatched bars show the groups or combination of groups being compared

	Number of patients	P3NP concentration (ng/ml)*	
		Median	Range
Controls			
Normal controls	18	5·4	(2·0–11·[illegible])
Psoriatic controls	18	7·4	(3·3–11·[illegible])
Methotrexate treated			
Normal	13	10·6	(6·9–17·7)
Steatosis	7	12·5	(7·5–19·4)
Inflammation without fibrosis	18	13·8	(6·3–27·[illegible])
Fibrosis	10	14·8	(10·4–26·5)
Cirrhosis	3	17·0	(10·2–46·4)



* Normal range 4–12 ng/ml. NS, not significant.

two groups ($P > 0{\cdot}05$ Mann–Whitney U-test) (Table 1). Serum P3NP levels were abnormal in 35 (69%) of the methotrexate-treated patients: levels were significantly elevated above controls ($P < 0{\cdot}0001$ Mann–Whitney U-test), even in those patients with normal histology or steatosis alone ($P < 0{\cdot}0001$ Mann–Whitney U-test) (Fig. 1); levels in patients with undisputed liver damage (inflammation, fibrosis and cirrhosis) were significantly higher than in patients with normal histology or steatosis ($P < 0{\cdot}05$ Mann–Whitney U-test) (Table 1).

There was no correlation between serum P3NP levels and either the cumulative dose of methotrexate or the duration of treatment (Spearman correlation coefficients, $-0{\cdot}0265$, $n = 51$, $P > 0{\cdot}05$ and $0{\cdot}0309$, $n = 51$, $P > 0{\cdot}05$ respectively).

Liver function

All patients with normal liver histology or steatosis alone had normal serum aminotransferases. Only seven patients had elevated serum ALT and these were patients with inflammation or fibrosis on biopsy. Three patients (with inflammation, fibrosis and cirrhosis) had marginally raised AST values. One patient with cirrhosis had a serum GGTP level of 179 U/l. All patients had normal bilirubin levels.

There was no significant correlation between histological grade and either serum ALT or bilirubin values, but statistically significant correlations between histological grade and AST and GGTP were found (Spearman correlation coefficient, $0{\cdot}4178$, $n = 52$, $P < 0{\cdot}005$ and $0{\cdot}4681$, $n = 51$, $P < 0{\cdot}005$ respectively). However, as stated above, most of the standard serum liver function test values were within the normal range.

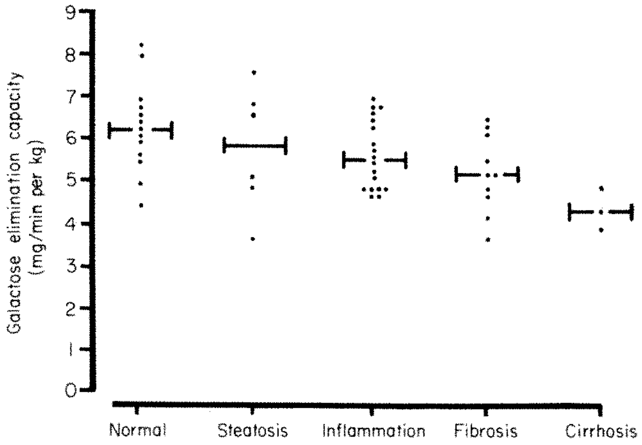


FIGURE 2. Galactose elimination capacity in methotrexate-treated psoriatics divided according to histological status. Medians are indicated by bars. The lower limit of normal for GEC is 4·7 mg/min per kg (mean – 2SD).

Dynamic liver function tests

GEC was estimated in 50 patients. Overall 14% had abnormally low GEC values (< 4·7 mg/min per kg) and GEC decreased with increasing severity of liver damage (Spearman correlation coefficient, –0·4249, $n = 50$, $P < 0{\cdot}005$) (Fig. 2).

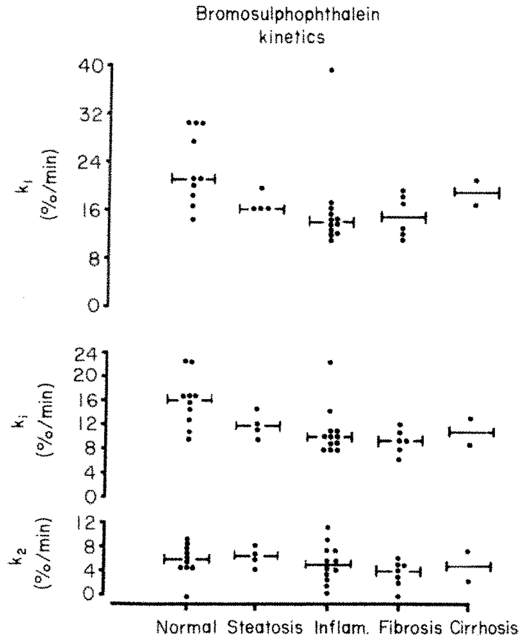


FIGURE 3. Bromosulphophthalein kinetics in methotrexate-treated psoriatics divided according to histological status. Medians are indicated by bars. The lower limits of normal are (mean – 2SD): BSP-k_i, 9·4%/min per kg; BSP-k_1, 11·3%/min per kg; BSP-k_2, 1·5%/min per kg.

BSP elimination kinetics were determined in 35 patients. BSP-k_1 was above the lower limit of normal in all patients; BSP-k_i was abnormally low in 15% and BSP-k_2 in 9% of patients (Fig. 3). A significant decrease in BSP-k_1, BSP-k_i and BSP-k_2 was observed with increasing liver damage (Spearman correlation coefficients, $-0{\cdot}4709$, $n = 34$, $P < 0{\cdot}01$; $-0{\cdot}5914$, $n = 34$, $P < 0{\cdot}001$ and $-0{\cdot}3931$, $n = 35$, $P < 0{\cdot}05$ respectively) (Fig. 3).

GEC correlated with BSP-k_i (Spearman correlation coefficient, $0{\cdot}3707$, $n = 34$, $P < 0{\cdot}05$) but not with BSP-k_1 or with BSP-k_2 (Spearman correlation coefficients, $0{\cdot}2693$, $n = 34$, $P > 0{\cdot}05$ and $0{\cdot}2444$, $n = 35$, $P > 0{\cdot}05$ respectively). Serum P3NP levels were negatively correlated with BSP-k_1, BSP-k_i, BSP-k_2 and GEC (Spearman correlation coefficents, $-0{\cdot}3993$, $n = 34$, $P < 0{\cdot}05$; $-0{\cdot}5007$, $n = 34$, $P < 0{\cdot}005$; $-0{\cdot}5074$, $n = 35$, $P < 0{\cdot}005$ and $-0{\cdot}2958$, $n = 50$, $P < 0{\cdot}05$ respectively).

DISCUSSION

A quarter of our psoriatic patients had fibrosis or cirrhosis, a proportion which emphasizes the need for a reliable and sensitive test to detect liver damage.

A highly significant elevation of serum P3NP levels was found in our methotrexate-treated patients compared to the control group of psoriatics who had never received methotrexate. As the control group of psoriatics had normal levels of P3NP similar to normal controls without psoriasis, we can attribute the elevated P3NP levels to the effects of the methotrexate rather than to the effects of active psoriasis *per se*, i.e. the raised P3NP levels probably derive from the liver and not from the skin.

The exact processes resulting in increased serum P3NP levels following liver damage are not known. The raised levels may be due solely to increased collagen synthesis, or inflammation and tissue remodelling may release P3NP from intact procollagen molecules normally present in the liver matrix. In our sudy, as in a previous smaller study,[7] a wide range of P3NP values were found in each histological group. Normal levels were found in only three of the 13 patients with fibrosis or cirrhosis, and raised levels were present in half the patients with steatosis or normal histology. As P3NP levels reflect biochemical turnover at the moment at which the sample is taken, an explanation of normal P3NP levels in the presence of histological damage is that the process producing the damage is reduced or inactive at the time of sampling.

It could be argued that the raised P3NP level seen in those patients with normal histology is due to a false negative liver biopsy. However elevated serum P3NP may reflect early damage not visible on light microscopy; electron microscopic studies are underway to see if there is evidence of ultrastructural damage in these patients.

Since completion of our study, Risteli *et al.*[8] have published data on serum P3NP levels in 24 methotrexate-treated psoriatics. A similar proportion of their fibrotic and cirrhotic patients had elevated levels of P3NP, but only one of their patients with normal histology had a raised value. The two studies cannot be compared directly, as P3NP assays with different specificities were used. It is apparent, however, that the Risteli assay does not detect the early changes in P3NP levels seen in our patients with apparently normal liver biopsies.

While P3NP was abnormal in half of all our methotrexate-treated patients, and in three-quarters of those with fibrosis or cirrhosis, the conventional and dynamic liver function tests were abnormal in only a quarter of our study patients and are thus of little practical value. This confirms previous observations that the conventional and dynamic liver function tests are poor guides to the development of fibrosis in methotrexate treatment.[9-11] In other liver diseases dynamic liver function tests are used as serial measurements to monitor changes as diseases progress, and as such, may be of use in methotrexate-treated patients. These tests, however, are expensive and time-consuming to perform.

The predictive value of sequential serum P3NP estimations in psoriatic patients receiving methotrexate is now being evaluated. Electron microscopic studies currently being undertaken may help us to establish whether the elevated serum P3NP levels which we observed in some patients with normal biopsies at the light microscopic level are in fact indicative of active fibrosis at the earliest stages. If this can be shown, measurement of serum P3NP levels may allow us to identify those patients most likely to develop significant liver damage as a result of methotrexate treatment.

ACKNOWLEDGMENTS

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effect of photochemotherapy with trimethylangelicin on murine cutaneous immune cells

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SUMMARY

The effect of photochemotherapy with 4,4′,6 trimethylangelicin (TMA), a new monofunctional furocoumarin with a high antiproliferative activity, was studied on murine dendritic epidermal cells. Female mice (C3H/HeN[MTV$^-$]) were treated topically with the drug three times a week for 4 consecutive weeks followed each time by 1 J/cm^2 of UVA radiation. At the end of the treatment almost total depletion of ATPase$^+$, Ia$^+$ and Thy-1$^+$ dendritic epidermal cells was observed, associated with marked hyperpigmentation but no gross or microscopic phototoxicity. Although 4,4′,6 TMA is not phototoxic in mice, it affects the cutaneous immune cells.

The new furocoumarin, 4,4′,6 trimethylangelicin (TMA), has a high capacity to form monofunctional adducts in DNA.[1] It also has a high antiproliferative activity, but produces little or no phototoxicity in the skin. It is, therefore, an attractive candidate as a new agent for use in photochemotherapy.[2] We have assessed the effects of 4,4′,6 TMA on immune cells that reside in the epidermis of mice.

METHODS

Mice

Specific pathogen-free C3H/HeN (MTV$^-$) female mice were obtained from the NCI-Frederick Cancer Research Facility (Frederick, MD, U.S.A.). The mice were 9 weeks old when used in these studies and there were six mice in each experimental group.

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Drug

4,4′,6 TMA was dissolved in 70% ethanol to form a 0·125% w/v solution. shielded from light.

Radiation source

UVA radiation was provided by a Dermalight 2001 using an optical H1 filter (Derma Systems, Studio City, CA, U.S.A.). The lamp was rotated horizontally and the mice we placed 22 cm below in a plastic cage separated into individual chambers. The cage was covered with a wire mesh. The spectral irradiance was measured under the mesh by a calibrated IL-700A research radiometer (International Light, Inc., Newburyport, MA, U.S.A.) using a UVA detector. The irradiance value was 5·63 mW/cm^2 and 99·5% of the spectral output of the lamp was between 320 and 400 nm, with the peak emmission occurring at 366 nm.

Treatment

Treatment was given three times a week for 4 consecutive weeks. The dorsal fur was removed with electric clippers and 250 μg 4,4′,6 TMA was applied to the back. Forty-five minutes later the mice were irradiated with 1 J/cm^2 UVA. In the three control groups, one received TMA alone, one UVA alone, and one no treatment at all. Twenty-four hours after the 1st, 6th and 12th treatments, mice from each group were killed and the treated skin excised.

Staining procedures

The epidermis was removed from the dermis after a 2-h incubation at 37°C in buffered EDTA.[3] For ATPase staining, the epidermal sheets were fixed for 1 h at 4°C in 0·05 M cacodylate-2% paraformaldehyde solution. The staining was performed as described previously.[4] Indirect immunofluorescence was used to demonstrate Ia$^+$ and Thy-1$^+$ dendritic epidermal cells.[4] Briefly, after 30-min fixation with acetone, the epidermal sheets were refrigerated overnight with either mouse anti-Ia$^+$ or rat anti-mouse Thy1·2 monoclonal antibodies (Becton Dickinson, Mountain View, CA, U.S.A.). As their respective second antibodies, fluorescein-conjugated F(ab′)$_2$ fragments of goat anti-mouse IgG and the rhodamine-conjugated IgG fraction of goat anti-rat antibodies were used (Cooper Biomedical, Inc., Malvern, PA, U.S.A.).

Cell counts

Between 10 and 20 fields from each experimental group were counted at × 400 magnification using a Nikon Optiphot microscope with a calibrated ocular grid. The results are expressed as percentages of the number of cells in the untreated control mice.

Histology

Skin samples from each experimental group were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin.

Statistics

Student's 2-tailed t-test was used to test the significance of differences between control and treated groups.

RESULTS

Phototoxicity and pigmentation

Before each treatment, the mice were observed for phototoxicity. During the entire treatment period, no gross phototoxicity was observed in mice treated with 4,4′,6 TMA plus UVA or UVA alone. Intense hyperpigmentation of the skin was observed at the end of 4 weeks at sites treated with TMA plus UVA. Histological examination of the treated skin revealed no evidence of inflammation. The only changes observed were hyperkeratosis and acanthosis with increased pigmentation in the basal and upper layers of the epidermis (Fig. 1).

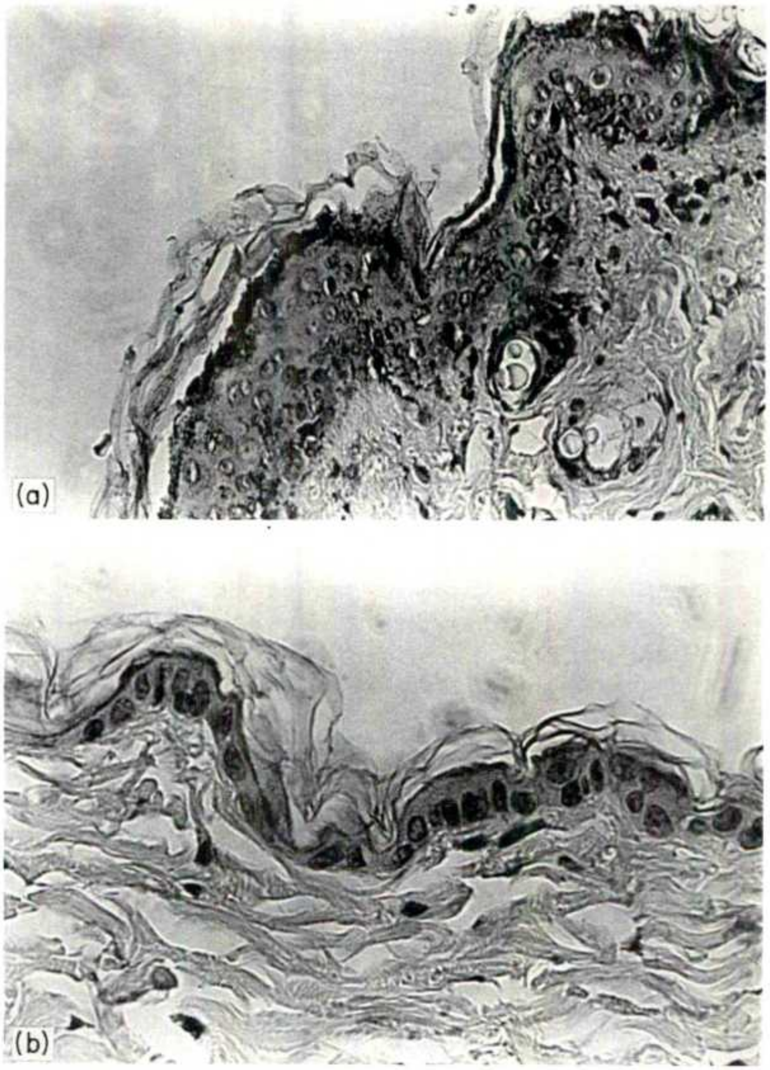

FIGURE 1. (a) Hyperkeratosis, acanthosis and increased pigmentation in skin treated for 4 weeks with 4,4′,6 trimethylangelicin plus UVA radiation (Hematoxylin and eosin, × 180). (b) Normal morphology of skin treated for 4 weeks with 4,4′,6 trimethylangelicin alone. (Hematoxylin and eosin, × 360).

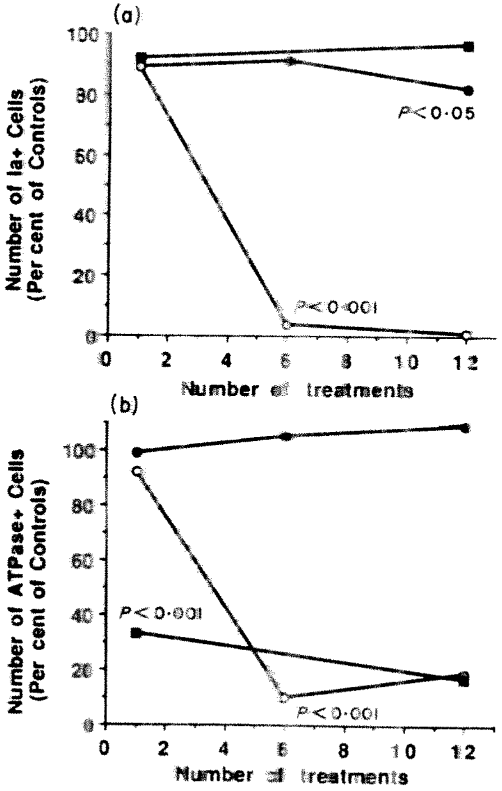

FIGURE 2. Effect of 4,4′,6 trimethylangelicin (TMA) plus UVA radiation on the number of Ia$^+$ (a) and ATPase$^+$ (b) Langerhans cells. The mean numbers (± SEM) of Ia$^+$ cells per mm^2 in untreated mice after 1st, 6th, and 12th treatment were 817 ± 53, 697 ± 52 and 816 ± 28 and that of ATPase$^+$ cells were 880 ± 38, 794 ± 60 and 807 ± 45 respectively. ●, TMA; ○, TMA + UVA; ■, UVA.

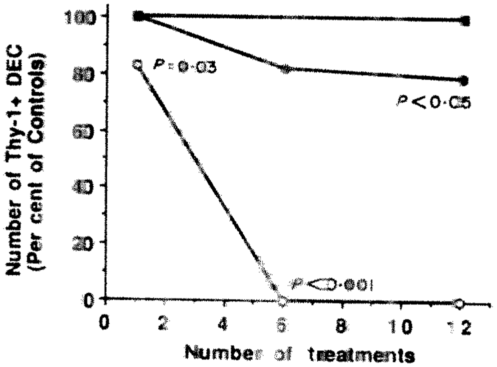

FIGURE 3. Effect of 4,4′,6 trimethylangelicin (TMA) plus UVA radiation on the number of Thy-1$^+$ dendritic epidermal cells (DEC). The mean numbers (± SEM) of Thy-1$^+$ DEC per mm^2 in untreated mice after 1st, 6th and 12th treatment were 477 ± 26, 502 ± 37 and 502 ± 18 respectively. ●, TMA; ○, TMA + UVA; ■, UVA.

Effect on dendritic epidermal cells

Although 4,4′,6 TMA plus UVA had no effect on the number of ATPase$^+$ and Ia$^+$ cells after a single treatment (Fig. 2), it did significantly ($P = 0{\cdot}03$) deplete Thy-1$^+$ dendritic epidermal cells (dEC), which following six treatments were totally absent (Fig. 3). At the end of the experiment (12 treatments) there was almost total depletion of Ia$^+$ and ATPase$^+$ cells from the epidermis. 4,4′,6 TMA alone had no effect on the number of ATPase$^+$ cells. However, for Ia$^+$ and Thy-1$^+$ dEC a small but significant depletion was seen with the drug alone as compared to the untreated group at the end of the experiment. After 4 weeks of treatment more ATPase$^+$ cells than Ia$^+$ cells were seen in the treated skin ($P < 0{\cdot}001$). The effect of UVA radiation alone was examined after the first and last treatments. UVA alone did not deplete Thy-1$^+$ and Ia$^+$ cells but significantly depleted ATPase$^+$ cells.

DISCUSSION

Since the introduction of photochemotherapy as a treatment of psoriasis in 1974,[5] it has been shown that 8-methoxypsoralen (8MOP) plus UVA radiation alters immune cells in the epidermis.[6,7] This bifunctional furocoumarin has, in addition to its effects on cutaneous immune cells, marked cutaneous phototoxicity when followed by UVA radiation. Since 8MOP has undesirable side-effects, such as phototoxicity and skin cancer induction, efforts have been made to find compounds that will have fewer side-effects without loss of therapeutic potency. Recently, we found that angelicin, a prototype of monofunctional furocoumarins, plus UVA radiation also decreased the numbers of Langerhans cells and Thy-1$^+$ dEC.[4]

The new drug 4,4′,6 TMA is the most active monofunctional furocoumarin identified to date in terms of its photobinding to DNA, being four times as photoreactive with DNA as 8MOP.[1] Our study indicates that 4,4′,6 TMA followed by UVA radiation depletes ATPase$^+$, Ia$^+$, and Thy-1$^+$ dEC or their markers from the epidermis, despite the complete absence of cutaneous phototoxicity. In addition, fewer Ia$^+$ cells were detected than ATPase$^+$ cells after 4 weeks of treatment, suggesting that the Ia marker is more sensitive to this type of photochemotherapy than the ATPase marker. This same differential sensitivity was observed with angelicin treatment.[4] We conclude that 4,4′,6 TMA plus UVA radiation has the same ultimate effects as 8MOP plus UVA radiation on the phenotypic markers of cutaneous immune cells. The marked hyperpigmentation observed after 4 weeks of treatment, without accompanying phototoxicity, suggests that this drug would be useful in the treatment of vitiligo.

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Cornified envelopes in congenital disorders of keratinization

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SUMMARY

A morphological and biochemical analysis was made of cornified envelopes isolated from patients with different congenital disorders. Nomarski contrast microscopy of the envelopes showed that their morphology was not greatly altered in several types of keratoderma and parapsoriasis, but it was grossly modified in ichthyotic disorders. The various types of ichthyoses, keratoderma palmoplantare, KID syndrome and parapsoriasis showed, after cyanogen–bromide cleavage, peptide patterns similar to those obtained from healthy subjects. In contrast, envelopes from patients with Darier's disease, congenital pachyonychia and erythrokeratoderma variabilis showed markedly different peptide patterns.

The cornified envelope (CE) is an insoluble protein structure synthesized during terminal differentiation of epidermal keratinocytes.[1] It is formed at the level of the upper granular layer by plasma membrane-associated transglutaminase.[2,3] This calcium-dependent enzyme catalyses the covalent cross-linking of precursor proteins via $\gamma\lambda$-glutamyl-ε-lysine isopeptide bonds. In the epidermis two morphologically distinct types of CEs have recently been identified:[4] a polygonal rigid type (CE_r) and an irregularly shaped type with a fragile appearance (CE_f) which, in healthy skin, is only present in the lower part of the stratum corneum. These two types possibly represent two successive steps of CE maturation. The stratum corneum of normal subjects contains mainly CE_r, whereas the scales in psoriasis contain a much higher proportion of CE_f. Keratinocytes in submerged cultures are only able to form CE_f.[4]

Several CE precursor proteins have been identified in cultured keratinocytes.[5-7] These proteins are either cytosoluble, e.g. involucrin,[5] or plasma-membrane bound.[6] Involucrin has been proposed as the major precursor of cornified envelopes of cultured normal keratinocytes.[5] However, transformed keratinocytes (line SV-K14), which contain a very low level of involucrin, are nevertheless able to form CEs after being treated with a calcium ionophore, probably by using a different set of precursor proteins.[8] Moreover cyanogen–bromide peptide

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mapping of purified envelopes revealed differences in the composition of the CEs from cultured normal keratinocytes, SV-40-transformed keratinocytes,[8] normal epidermis and psoriatic scales.[4] These results indicate that the molecular composition of the CE may not be strictly constant and may vary according to the availability of substrate proteins when the cross-linking enzyme transglutaminase becomes activated. We have analysed the CEs from patients with various congenital hyperkeratotic lesions.

METHODS

Patients

Keratosis follicularis (Darier's disease). Five patients, aged 16–62, with typical lesions on trunk and legs.

Keratoderma palmoplantare. Three patients, aged 20–52, with lesions only on soles and palms.

KID (keratitis, ichthyosis, deafness) syndrome. A girl, aged 10, with keratoderma palmoplantare and red ichthyotic lesions especially on the face, ears and extremities.

Congenital pachyonychia. A boy, aged 15, with marked changes of nails and hair and severe keratoderma of the soles and palms.

Erythrokeratoderma variabilis. A woman, aged 21, with widespread areas of erythema and ichthyosis that she has had since birth.

Parapsoriasis. Two patients, aged 61 and 81, with circumscribed, oval, slightly scaly and persistant erythematous lesions.

Ichthyosis vulgaris. Three patients, aged 30–37, with dry skin and marked ichthyotic lesions on the extremities.

Ichthyosis, brittle hair syndrome. Two brothers, aged 16–18, with dry ichthyotic skin and brittle hair since birth. The stature was normal and an amino acid analysis had not yet been performed to exclude a diagnosis of trichothiodystrophy.

These diagnoses were confirmed both clinically and histologically.

Collection of the samples

The patients had no treatment for at least 2 months before sampling apart from moisture creams. The corneocytes were collected from the stratum corneum by curettage and the material obtained placed in an aqueous solution of 2% sodium dodecyl sulphate (SDS), 0·1% dithioerythritol and 0·01% sodium azide and stored at 4°C.

Cornified envelope purification

Purification was performed as previously described.[4] The samples were boiled with vigorous stirring for 10 min. The CEs were recovered with 5 min centrifugation at 3500 ***g*** and resuspended in the above described mixture. Boiling and centrifugation was repeated four times and the final pellet was suspended in an equivalent volume of Laemmli sample buffer[9] and transferred into a dialysis bag (Spectra/por 6/132542; M_r cut off: 50,000; Spectrum Medical Industries, Los Angeles, CA, U.S.A.). The dialysis bag was immersed into an electrophoretic tank containing Laemmli running buffer and subjected to electrophoresis at 20–50 V for 24 h. The envelopes were recovered by centrifugation and the electrodialysis procedure was repeated three more times.

Cyanogen bromide (CNBr) cleavage of CEs

The protein content of the envelopes was determined by the Lowry procedure[10] after digestion with proteinase K.

The envelopes were suspended in 70% (v/v) formic acid, and 20 mg of CNBr was added per mg protein. Under these conditions, selective cleavage of peptide bonds occurs at the level of methionine residues. After 24 h of agitation at room temperature the mixture was diluted 1:5 with distilled water. After drying under a vacuum the residues were suspended in Laemmli sample buffer.

Electrophoresis

Electrophoresis of the peptides was performed according to the procedure of Laemmli.[9] Fifteen per cent polyacrylamide gels were used and 10 μg of protein were loaded per lane. The peptides were revealed by silver staining.[11]

RESULTS

Morphology of the CEs

The morphological aspects of the CEs obtained from patients affected with different dermatoses are presented in Figure 1. As a reference, CEs from normal human epidermis are shown in Figure 1a. As described[8] they mainly consisted of CE_r with only a minor proportion of CE_f. Due to the limited number of patients examined, it is not possible to determine precisely the proportion of type CE_r and CE_f in each dermatosis. As a consequence, the different diseases are described in qualitative rather than in quantitative terms.

The morphology of CEs obtained from patients with Darier's disease is shown in Figure 1b; being mainly composed of CE_r. However, their size and shape are more heterogeneous than those from normal skin.

Palmoplantar keratoderma lesions (Fig. 1c) contained mainly CE_r. The envelopes were larger than those from normal epidermis. CEs prepared from a patient with KID syndrome (Fig. 1d) and from a patient with congenital pachyonychia (not shown) were very similar to those of normal skin apart from a relative increase in CE_f. CEs from a patient with erythrokeratoderma variabilis (Fig. 1e) are similar to those from normal skin.

In parapsoriatic epidermis (Fig. 1f) the type CE_r is predominant. A characteristic feature of this disease was the presence of a nuclear-like inclusion in some envelopes which made it easy to distinguish it from psoriasis vulgaris (Fig. 1h). Even though parakeratosis is also a characteristic feature of psoriasis, there were no nuclei present in the CE preparation (Fig. 1h), indicating differences in the cross-linking reaction in both diseases.

The CEs from ichthyosis vulgaris (Fig. 1g) contained an increased number of type CE_f and those from one ichthyotic patient were reduced in size. This was also observed in the samples prepared from the two patients with ichthyosis, brittle hair syndrome.

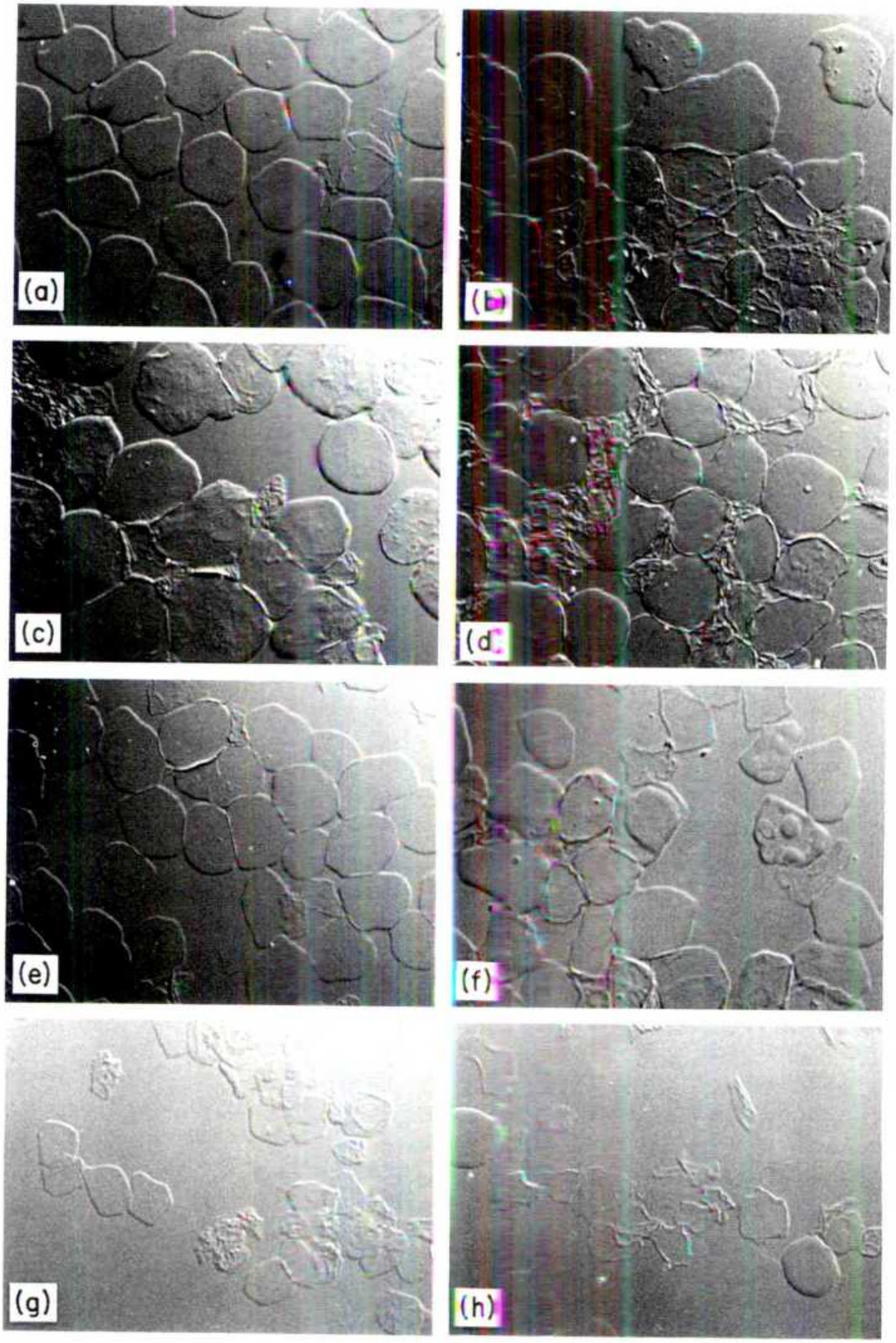

FIGURE 1. Nomarski contrast micrographs (× 120) of the cornified envelopes obtained from normal epidermis (a) and from the lesions of patients with Darier's disease (b), keratoderma palmoplantare (c), KID syndrome (d), erythrokeratoderma variabilis (e), parapsoriasis (f), ichthyosis vulgaris (g) and psoriasis vulgaris (h).

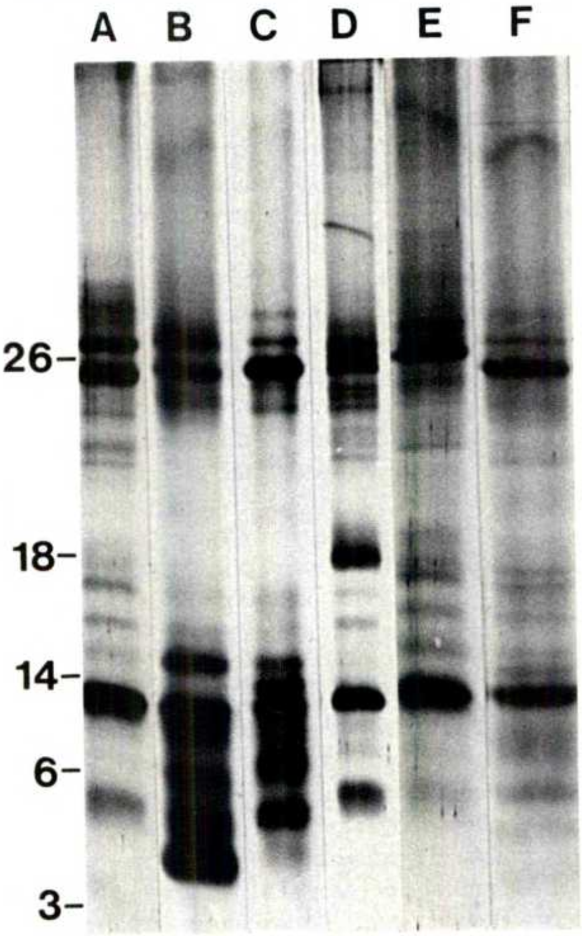

FIGURE 2. Cyanogen-bromide peptide mapping of the cornified envelopes obtained from normal epidermis (A) and from the lesional skin of patients with Darier's disease (B), congenital pachyonychia (C), erythrokeratoderma variabilis (D), ichthyosis vulgaris (E) and keratoderma palmoplantare (F). The position of molecular weight markers (in kDa) is indicated.

CNBr peptide mapping

The CNBr peptide maps of the CEs obtained from different origins are presented in Figure 2. The peptide pattern of CEs purified from the lesional skin of patients with Darier's disease differed markedly from those of normal epidermis, in particular at the level of peptides of molecular weight comprised between 18 and 6 kDa (map B to be compared with map A). A similar difference was also observed in congenital pachyonychia (map C).

The CNBr peptide maps of CEs obtained from a patient with erythrokeratoderma variabilis (map D) exhibited a characteristic peptide of molecular weight 18 kDa not present in the peptide pattern of CEs from normal skin.

The peptide maps of CEs obtained from the patients with ichthyosis vulgaris (map E), palmoplantar keratoderma (map F) and of the other diseases studied (not shown) were not significantly different from those of healthy subjects.

DISCUSSION

According to the morphology of the CEs, congenital disorders of keratinization can be divided into two classes. One comprises keratoderma palmoplantare, KID syndrome, congenital pachyonychia, erythrokeratoderma variabilis and parapsoriasis in which the morphology of the CEs is very close to that of normal skin. In the other class, that consisted of Darier's disease, ichthyosis vulgaris and the ichthyosis brittle hair syndrome, the morphology of the CEs is grossly altered. In the ichthyotic-like dermatoses the morphology of the CEs resemble closely that of psoriatic epidermis (Fig. 1g to be compared with Fig. 1h) and contain an increased number of CE_f. The reduced size of CEs observed in some ichthyotic patients is apparently correlated with a reduced size of corneocytes.[12] In Darier's disease, the morphology of the CEs is heterogeneous, which could indicate that in this disorder the keratinocyte morphology is already altered at the level of the upper granular layer, in which the CE is formed.

The CNBr peptide mapping of CEs revealed that the peptide composition of CEs from patients affected with Darier's disease, congenital pachyonychia and erythrokeratoderma variabilis are different from those of normal skin. The pattern of CEs from patients with Darier's disease and congenital pachyonychia showed an accumulation of low molecular weight peptides similar but not identical to that observed in CEs from psoriatic patients.[8] The CE peptide pattern from a patient with erythrokeratoderma is strikingly different from that of normal and psoriatic individuals. The peptide patterns obtained with CEs from the other diseases resemble those of normal skin.

The morphology and peptide composition of the CEs are not strictly correlated. Ichthyosis vulgaris, for example, shows abnormal CE morphology with a normal peptide pattern, whereas congenital pachyonychia presents a normal CE morphology but an abnormal peptide pattern. This shows that the morphology of CEs does not depend exclusively on the peptide composition, but may also be affected by other parameters such as the attachment of the recently described ester-linked hydroxyacyl sphingosine.[13]

Biochemical studies of congenital disorders of keratinization have been limited so far to the analysis of keratin proteins. Bowden *et al.*[14] found normal keratin expression in ichthyosis but an alteration of the normal keratin pattern in Darier's disease. Expression of normal keratin subunits in ichthyosis was confirmed by the immunoblotting technique with AE1 and AE3 monoclonal antibodies.[15] These results on keratin expression in diseases cannot be strictly correlated with our findings on the CE peptide composition. However, not only the keratin subunits but also the CE peptide patterns of ichthyotic patients are identical to those of normal subjects.

The results show that different dermatoses can be distinguished by analysing either the morphological shape or the peptide pattern of the CEs, but further work is needed to evaluate the use of CE analysis in the diagnosis and classification of dermatological disorders.

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The effect of bacterial products on human fibroblast and keratinocyte detachment and viability

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SUMMARY

An *in vitro* model has been developed to study the effect of soluble bacterial products on the viability and detachment of skin cell types utilized for cultured grafts. Microbial products prepared from clinical isolates of bacterial species which most commonly colonize burn lesions showed marked variation in their ability to detach and kill both keratinocytes and fibroblasts. All three isolates of *Acinetobacter* spp. tested were effective in causing detachment and death of keratinocytes and fibroblasts, whereas *Escherichia coli, Proteus mirabilis* and *Enterobacter* spp. tested had little, or no, effect on detachment or viability for either skin cell type. Four *Staphylococcus aureus* isolates elicited variable strain-dependent results with regard to detachment and viability. One isolate possessed activity specific for keratinocyte detachment and death. These results indicate the possible undesirable effects such bacterial species may have on graft success in colonized burn wounds.

Recent developments in the culture of human skin keratinocytes have led to the prospect of improved management of burn wounds. This is particularly appropriate for patients with large, full thickness, body surface area burns, who have a high mortality. There are two techniques presently in use; cultured keratinocyte sheets[1,2] and skin equivalents, which consist of a fibroblast-colonized collagen lattice combined with a cultured keratinocyte epithelium.[3] The success rate has been variable, even when autologous cells have been used to avoid immunological rejection,[4] and in granulating wounds the graft is less likely to take[4] as they are inevitably colonized by micro-organisms.[5] The failure of the grafts may be due to the effects of bacteria on the cultured skin.

As a first step in determining whether the micro-organisms colonizing the wound could directly affect graft-take, an *in vitro* model was used to study the effect of soluble products of these bacteria on the detachment and viability of skin cells used for the cultured grafts.

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METHODS

Bacteria

All bacteria were clinical specimens freshly isolated from burn wounds. The organisms used in this study were *Pseudomonas aeruginosa* 1 and 2, *Staphylococcus aureus* 1–4, *Proteus mirabilis* 1 and 2, *Escherichia coli* 1 and 2, *Acinetobacter* spp. 1–3 and *Enterobacter* spp.

Tissue culture

Keratinocytes (SVK14) from an SV40 transformed cell line (provided by Dr I. Leigh, London Hospital, Whitechapel), and fibroblasts prepared from human foreskins were regularly maintained in DMEM (Gibco Ltd, Uxbridge, U.K.) supplemented with 10% (w/v) fetal bovine serum (Gibco Ltd.) and 0·375% (w/v) bicarbonate buffer. The cells were incubated in a 5% (v/v) CO_2 atmosphere in modular incubators (Flow Laboratories Ltd, Irvine, Scotland, U.K.) at 37°C.

Preparation of freeze-dried culture filtrates

One colony of each isolate grown on nutrient agar (Oxoid Ltd, Basingstoke, U.K.) was transferred to 30 ml of 18 amino-acid medium plus vitamins and salts, pH 7·4 in a 250 ml conical flask.[6] These cultures were incubated at 37°C on an orbital shaker (160 r.p.m.) for 18 h. Ten millilitres of each culture was transferred to 100 ml of fresh 18 amino-acid medium in a 2 l conical flask and incubated at 37°C and 150 r.p.m. for a further 8 h. Supernatants were prepared by centrifugation of the cultures at 10,000 ***g*** for 20 min. The pH of the supernatants was adjusted to pH 7·4 prior to filtering (0·2 μm pore diameter, Millipore Ltd, Watford, U.K.) into dialysis bags. The filtrates were dialysed at 4°C, three times in 10 volumes of distilled water. On completion of dialysis the filtrates were again filtered (0·2 μm pore diameter) and freeze-dried. The freeze-dried preparations were stored at −20°C. Immediately prior to assaying for their effect on cell detachment and cytotoxicity, the freeze-dried supernatants were resuspended in 10 ml Hank's medium (Gibco Ltd,) with 10 mM Hepes buffer.

Detachment assay

Fibroblasts at a density of $2{\cdot}0 \times 10^5$ cells ml^{-1} and keratinocytes at a density of $1{\cdot}8 \times 10^5$ cells ml^{-1} were seeded into 25-well flat bottomed plates (Sterilin, Cel-cult, Hounslow, U.K.). These were incubated overnight at 37°C in an atmosphere of 5% (v/v) CO_2. The medium was aspirated from each well, the cell sheets washed with 1 ml Dulbecco A medium (Oxoid Ltd) and 0·4 ml of each of the resuspended filtrates added to the wells. The 18 amino-acid medium, uninoculated, filtered, dialysed, freeze-dried and resuspended in Hank's medium was used as a control. The cells were subsequently incubated at 37°C in a moist atmosphere for 5 h. The liquid phase (A) was aspirated from each well and stored at 4°C. The remaining cell sheets were trypsinized using 0·05% (w/v) trypsin with 0·02% (w/v) EDTA in Dulbecco A medium. The reaction was stopped by adding medium containing serum. Both suspensions were subsequently centrifuged at 150 ***g*** for 5 min. The cells were suspended in 0·5 ml formol saline and stored at 4°C. Total counts of cells in each sample were determined using a Coulter counter with a 100 μm aperture. The percentage cell detachment was expressed as: $A/A+B \times 100$. Five replicates for each test and control were performed.

Cytotoxicity assay

To determine the proportion of viable cells in each sample, a drop of the suspension was mixed with an equal volume of trypan blue (0·4% w/v). The number of viable cells (X) excluding the dye, and stained dead cells (Y) in 12 microscope fields (10 × objective) were counted immediately. The percentage cell death was expressed as: $X/X+Y \times 100$. Five replicates for each test and control were performed.

Analysis of results

Percentage values were arcsine transformed and a two-way analysis of variance was performed. *A posteriori* multiple comparisons of means were carried out using the T-method.[7] Following analysis all data was back-transformed to percentages. The Spearman rank correlation coefficient was calculated for both cell types to determine whether there was a correlation between cell death and detachment.

RESULTS

The results are shown in Figures 1 and 2 for the effects of bacterial culture filtrates on fibroblast and keratinocyte detachment and viability. Cell detachment was effected by the bacterial culture filtrates, and the response was dependent on the filtrate ($P < 0{\cdot}01$). The *Acinetobacter* spp. 1 and

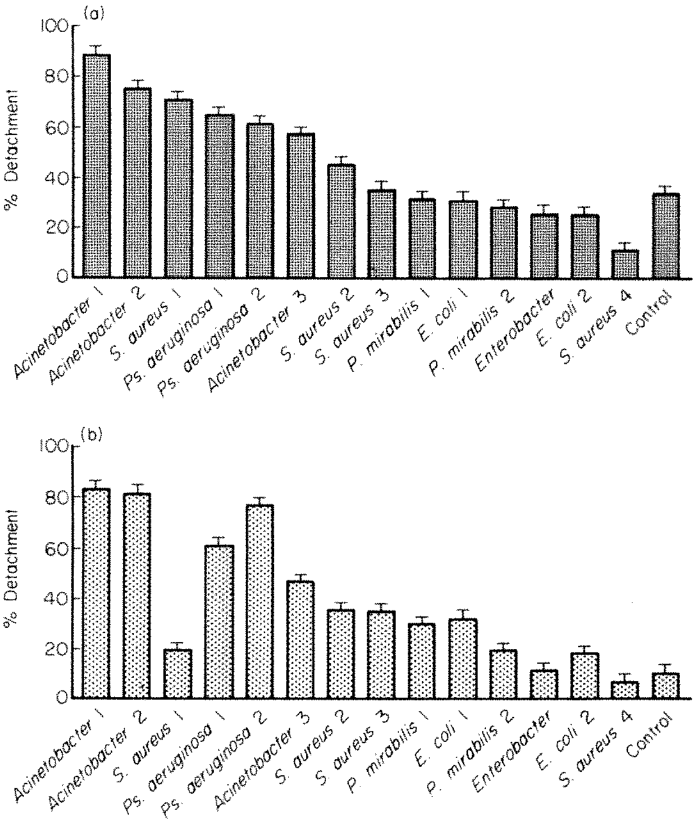

FIGURE 1.(a) The effect of various culture filtrates of bacteria on detachment of keratinocytes. The filtrates are arranged in rank order of their activity. (b) The effect of various culture filtrates of bacteria on detachment of fibroblasts. The filtrates are arranged in the order shown in (a).

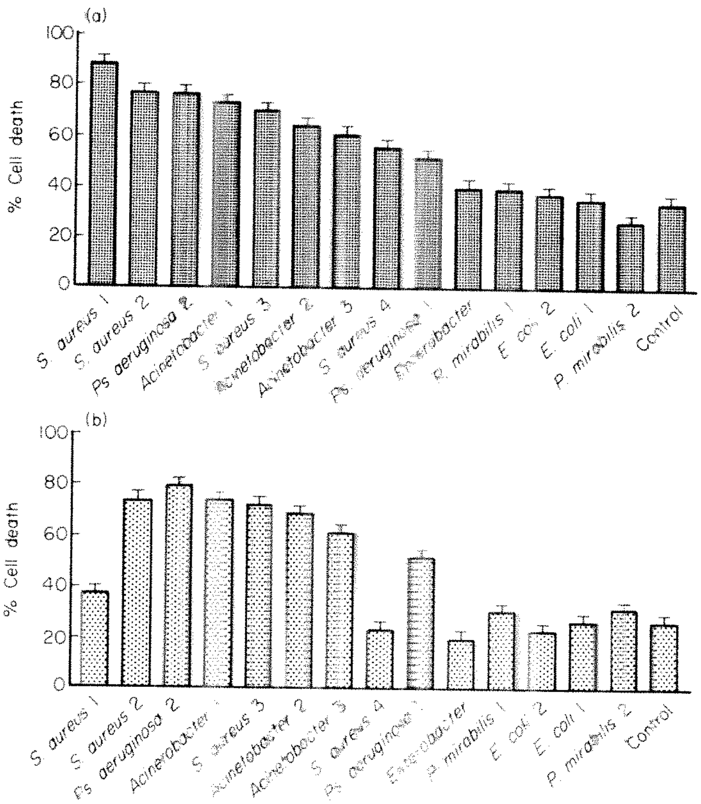

FIGURE 2.(a) The effect of various culture filtrates of bacteria on death of keratinocytes. The filtrates are arranged in rank order of their activity. (b) The effect of various culture filtrates of bacteria on death of fibroblasts. The filtrates are arranged in order shown in (a).

2 culture filtrates were the most potent in causing detachment of both keratinocytes and fibroblasts. *Escherichia coli*, *Proteus mirabilis* and *Enterobacter* spp. culture filtrates had no effect on detachment of keratinocytes compared to the control, and these filtrates caused little (*E. coli*, *P. mirabilis*) or no (*Enterobacter*) detachment of fibroblasts. Three of the four *S. aureus* culture filtrates caused fibroblast detachment and two *S. aureus* culture filtrates caused keratinocyte detachment. Of the 14 bacterial culture filtrates tested, seven of these caused significantly greater keratinocyte detachment than the control, and 11 of the filtrates had effects on the fibroblasts. There was a significant difference ($P < 0.01$) in detachment of the two cell types from the plastic substratum, keratinocytes detaching more than fibroblasts.

The effects of bacterial culture filtrates on cell death were similar to those on cell detachment, and this association was significant at the 95% confidence level. However, although the majority of cells which detached were non-viable, a small proportion were viable, and some non-viable cells remained attached to the plastic.

There was a significant effect of bacterial culture filtrates on cell death ($P < 0.01$). All the *S. aureus* isolate culture filtrates, except isolate 4, were cytotoxic to both keratinocytes and fibroblasts and *S. aureus* 1 was the most active. *Escherichia coli*, *Enterobacter* spp. and *Proteus* spp. culture filtrates were not cytotoxic compared to the control for either keratinocytes or fibroblasts.

DISCUSSION

The bacteria used in this study were isolated from burns patients and represent those most commonly colonizing and infecting burn lesions.[8] The fibroblast strain was isolated from normal human dermis and did not show any peculiarities in culture. Although the keratinocyte strain was a virus-transformed continuous-cell line, its ability to adhere to plastic showed no difference from normal human keratinocytes as assessed during routine culture and trypsinisation.

The control, dialysed bacteriological medium, had positive effects on cytotoxicity and detachment for both cell types. Preliminary experiments using tissue culture medium as the control gave no detectable effects. The micro-organisms growing in the bacteriological medium not only excreted products into it, but also depleted the medium of different components. This interaction may well explain why for example *S. aureus* 4 filtrate showed decreased keratinocyte detachment when compared with the dialysed bacteriological medium control.

The results of this *in vitro* investigation indicate that the bacteria can be placed into two groups, those whose soluble products do not directly affect fibroblast and keratinocyte detachment and viability and those which have direct effects. The harmful effects of the bacterial culture filtrates may help to explain graft failure when these bacteria are present on the graft-bed. If the detrimental effects of the bacteria are confirmed, this may contra-indicate the use of cultured grafts where there is heavy and specific colonization. Such a precaution is taken using traditional skin grafts if β-haemolytic *Streptococci* are present on the wound, since it is known that streptolysin, produced by these bacteria, break the fibrin bonding which is important in initial graft-take.[9] The *S. aureus* isolate 1 culture filtrate was specific for keratinocyte detachment and death. It is not yet known which component(s) in this filtrate, and in others with cell detachment and lethal activity, are responsible for the biological activity, though there are many extracellular toxins produced by bacteria that might be responsible.[10] Fader *et al.* showed that a 253 kDa toxin, with two subunits, was cytotoxic to foreskin cells.[11] They concluded, with limited evidence, that the cytotoxin was an aggregation of α-haemolysin with δ-haemolysin. Unfortunately in this study there was no information of the specific cell type or types used in the assays and no differentiation made between cell detachment and death. This is clearly important in the light of the results reported here.

The assay used in this work and by Fader *et al.* was based on skin cell culture on a plastic substratum. In future, experiments should be directed to the analysis of filtrates of cultures of burns bacterial isolates using cell lines cultured on collagen.

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The influence of area of application on sensitization by dinitrochlorobenzene

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SUMMARY

We have investigated the effect on sensitization of altering the area of application of 2,4,dinitrochlorobenzene (DNCB) at a constant dose per unit area. We showed that, when an area of less than 1 cm^2 is used, this area is critical in determining the degree of sensitization. This contrasts with previous work that showed, for larger areas, an alteration in the area of application had little effect on sensitization, whereas keeping the area constant and increasing the concentration of DNCB increased the degree of sensitization. We suggest that not only is the amount of antigen important in determining response, but also the distribution of the antigen as presented to the afferent limb of the immune system.

The characteristics and physiology of delayed type hypersensitivity in man are poorly understood and we cannot explain why, given equal exposure to an antigen, some individuals develop contact hypersensitivity while others do not. We have previously shown that when response is measured as an increase in skinfold thickness, sensitization by the potent antigen 2,4,dinitrochlorobenzene (DNCB) follows predictable log–dose response relationships.[1] Using this technique we have shown that the area of application, the amount of DNCB applied and the sex of the individual are important determinants of response.[2–4] In our previous work we found that when the area of the sensitizing dose of DNCB was kept constant, changes in the total amount of DNCB applied resulted in large differences in sensitization as reflected by the magnitude of the response to challenge 1 month later.[1] However, we later showed that if the concentration applied per unit area of skin was kept constant, changes in the area of application (and hence the total amount of DNCB applied) produced little change in sensitization, even though the efferent pathway was not saturated.[4] This suggested that the density of the antigen (perhaps on each antigen presenting cell) is also important in determining response, and not just the total amount of antigen applied. If this was to hold true over a wide range of areas then it should be possible in theory to reduce dose and area to an infinitesimal level and still obtain sensitization. This appeared improbable, and it seemed more likely that the degree of

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sensitization would be influenced by the area of application, at least at small areas. However, as the area of application is increased, the afferent system may become saturated and a change in sensitization would no longer be observed. In order to test this hypothesis we chose an area of application as small as was practicable to work with to see how sensitization was altered.

METHODS

Subjects

Volunteers were recruited from the dermatology out-patient department. Individuals with a history of atopy, contact dermatitis or inflammatory dermatoses were excluded. Ethical committee approval was obtained for this study and all subjects gave informed consent. No patients were included who were receiving any drugs or treatment known to interfere with the development or expression of contact sensitivity.

Experimental groups

Two groups of subjects were studied. In the first group (large area) 12 male and 15 female subjects were sensitized with 30 μg of DNCB applied to a standard 1 cm diameter patch-test felt (Al-test) of area 0·8 cm^2 held in place on the medial side of the thigh for 48 h. In the second group (small area) six male and nine female subjects were sensitized with 3 μg of DNCB placed on a modified Al-test pad of area 0·08 cm^2. The total amount of DNCB applied differed by a factor of 10 between the groups as did the area of application, but the concentration per unit area in both groups was the same (38 $\mu g/cm^2$). One month later, sensitivity was determined by measurement of responses to a graded series of challenge doses applied simultaneously: subjects were challenged with three doses of DNCB (8·8, 12·5, 17·5 μg) placed on Al-test pads (0·8 cm^2) on the inner aspect of the upper arm for 6 h. Skinfold thickness was measured twice at each site with modified Harpenden calipers immediately before and 48 h after challenge. The responses to challenge were expressed as change in skinfold thickness (final thickness minus initial thickness) and plotted against log-challenge dose. The results were compared using a split unit analysis of variance.

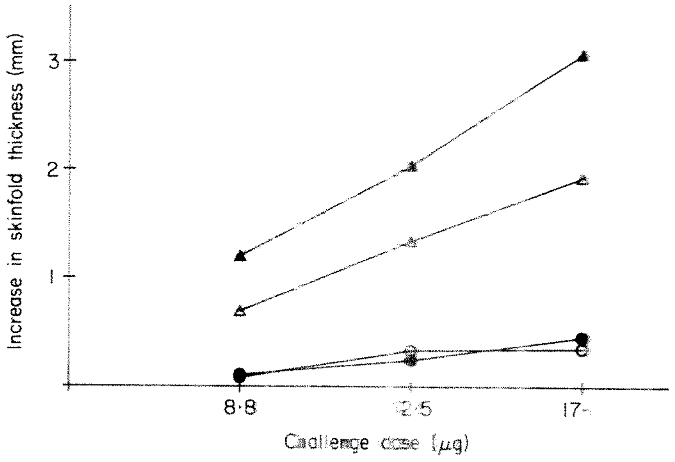

FIGURE 1. Responses to challenge with DNCB following sensitization with a constant concentration per unit area of DNCB (38 $\mu g/cm^2$) applied to either 0·8 cm^2 (large area) or 0·08 cm^2 (small area). ▲, female 0·8 cm^2; △, male 0·8 cm^2; ●, female 0·08 cm^2; ○, male 0·08 cm^2.

RESULTS

While there was no evidence of non-linearity in the log-dose response ($F_{3;76} = 0{\cdot}18$; $P = 0{\cdot}9$), there was strong evidence that the slopes were different in the four experimental groups ($F_{3;76} = 9{\cdot}8$; $P < 0{\cdot}001$). Most of this difference in response is accounted for by the greater responses seen in the large area groups of either sex (Fig. 1). The slopes in both small area groups were small (male mean 0·13 [95% confidence limits, −0·18, 0·45]; female 0·18 [−0·08, 0·43]). In the large area groups, there were substantial dose-responses (male 0·62 [0·39, 0·84]; female 0·93 [0·72, 1·13]), and as we have previously found[2] there was evidence of a difference between the sexes ($t_{76} = 2{\cdot}05$, $P < 0{\cdot}05$) with females showing a greater response at all challenge doses.

DISCUSSION

In an earlier study[4] we showed that when the area of application of antigen is kept constant, changes in concentration of antigen (and hence total dose of antigen applied) produce clear dose-related differences in sensitization. As more antigen is applied it is probable that a greater amount will penetrate and the signal to the afferent limb of the immune system will be that much larger. In humans it is thought that the major cell type in skin responsible for the presentation of antigen is the Langerhans cell (LC) and presumably an increase in antigen concentration results in more antigen being available for presentation by the LC. It is known in mice that there is a clear relationship between the amount of antigen applied to the skin and the number of antigen bearing dendritic cells appearing in the regional lymph node.[5] By contrast we also found previously that if the dose per unit area was kept constant (either 16 or 35 $\mu g/cm^2$) changes in the area of application of DNCB to human skin within the range of 1·8–14 cm^2 produced little change in the degree of sensitization.[4] This suggested that with a constant dose per unit area, a small area of antigen will produce the same effect as a much larger area, but the effect of the absolute area chosen needed to be investigated. The results of this study showed that the influence of changes in area does vary with the absolute area and with small areas a tenfold change in area of application (at concentration 38 $\mu g/cm^2$) from 0·08 to 0·8 cm^2 produced clear differences in sensitization. Thus, the degree of sensitization cannot be understood solely in terms of amount of the antigen applied, which has implications for the understanding of delayed hypersensitivity in, for example, routine patch testing. When small areas are sensitized alterations in the area of application of DNCB and hence of total DNCB applied produce clear differences in sensitization. When larger areas are used to sensitize, changes in area no longer appear to affect sensitization, suggesting that the afferent limb of the immune system is influenced not solely by the amount of antigen but also the distribution of antigen on its antigen presenting cells.

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Antigen presentation in patients with recrudescent orofacial herpes simplex virus infections

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SUMMARY

Recovery from epidermal herpes simplex virus (HSV) infection depends primarily on development of an effective cell-mediated immune response, possibly generated following antigen (Ag) presentation by epidermal cells (EC). The ability of EC to present HSV Ag was investigated in 12 subjects with occasional recrudescent facial HSV infections. All had circulating HSV specific antibodies and cell-mediated immunity to the virus. Peripheral blood mononuclear cell suspensions, depleted of antigen presenting cells (APC) by glass adherence and then enriched for T cells by adsorption on nylon wool columns, did not proliferate in response to HSV Ag. Both EC suspensions, prepared from suction blister roofs, and glass-adherent peripheral blood mononuclear cells (AC) preincubated with ultraviolet-inactivated HSV, reconstituted the T-cell proliferative response to HSV. EC were more efficient than AC at presenting HSV Ag to T cells. Depletion of $CD1^+$ cells from EC suspensions by cell sorting reduced their ability to present HSV Ag and augmentation of $CD1^+$ cell numbers supplemented it. Preincubation of EC or AC with monoclonal antibodies to major histocompatibility complex class II antigens DP, DQ or DR, blocked the lymphoproliferative response to HSV Ag. Evidence was obtained that cells co-ordinately expressing products of the DP, DQ and DR loci are involved in presentation of HSV Ag by both EC and AC.

Human $CD4^+$ helper T lymphocyte and delayed-type hypersensitivity (DH) T lymphocyte responses depend on presentation of antigen (Ag) in the context of major histocompatibility complex (MHC) class II Ag on the surface of antigen presenting cells (APC).[1] MHC class II molecules are products of at least three major gene loci designated DP, DQ and DR within the MHC on chromosome 6.

Langerhans cells (LC) are the main APC in the skin,[2] all of which bear CD1 Ag and most of which bear MHC class II Ags.[3,4] In normal skin there are few lymphocytes in the epidermis.[5] Foreign Ag may be carried into the dermis or to draining lymph nodes by LC for presentation to

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T lymphocytes.[2,6] However, keratinocytes and LC release cytokines, such as interferons[7] and epidermal-cell derived thymocyte-activating factor, the production of which is enhanced in many skin diseases.[8] These cytokines may then recruit lymphocytes into the epidermis where local Ag presentation could take place.[8,9]

In human recrudescent herpetic skin infection, the virus is normally confined to the epidermis and local immune responses consist mainly of early infiltration with CD4^{+} T cells and macrophages, followed by CD8^{+} T cells.[10] Work with murine experimental models suggests that local DH or helper T-cell and cytotoxic T-cell responses are important in limiting the extent of and clearing virus from skin lesions and also in controlling local nervous system infection.[11-14] DH- or T-helper responses may act by recruiting and activating effector cells such as macrophages and cytotoxic T cells early in epidermal HSV infection.[12-14] Natural immune mechanisms, such as natural killer-cell activity, may play a role before adaptive immunity is established[15] and antibodies (Ab) may help prevent the spread of HSV to the nervous system.[16]

Since HSV is predominantly an epidermal pathogen and recovery from herpetic infections depends heavily on cell-mediated immunity (CMI) it is of interest to investigate the APC function of epidermal cells (EC) for HSV. Human EC have been shown to be able to present HSV Ag to the autologous peripheral blood mononuclear cells (PBMC)[17] and T cells.[18] This MHC class II-restricted Ag presentation[19] is amplified by enriching EC suspensions for LC[20] and reduced by depleting EC of LC. Work in mice has also shown that EC can present HSV Ag.[21] We have confirmed that EC and glass-adherent PBMC (AC) can act as APC for HSV type 1 (HSV-1) in patients with recrudescent HSV infection, and have extended previous observations by investigating the role of MHC class II Ags in this process by blocking Ag presentation with murine monoclonal Abs to DP, DQ and DR Ags and by enrichment for DP^{+} AC and CD1^{+} EC using cell sorting.

METHODS

Patients

Twelve volunteers (six male, six female; age range 23-47, mean age 37 years) who suffered from less than one to six recrudescent facial HSV infections annually were investigated at times when lesions were not present. They were either referred to the Dermatology Department at the Royal Infirmary of Edinburgh because of HSV or were staff members. All patients were in good general health and had circulating HSV-specific Ab measured by enzyme-linked immunosorbent assay (ELISA) and CMI to HSV indicated by *in vitro* lymphoproliferation to HSV Ag.[22]

Preparation of enriched T cells and AC from blood

PBMC were purified from fresh heparinized blood, washed and resuspended in RPMI 1640 medium (Gibco Ltd., Paisley, U.K.) containing 15% autologous plasma and antibiotics (culture medium), as described previously.[22] Two populations of PBMC were prepared as follows: PBMC were incubated on glass for 1 h at 37°C after which the non-adherent cells were washed off and the AC removed using a cell scraper (Costar Corporation, Cambridge, MA., U.S.A.) and vigorous pipetting. Non-adherent cells were incubated on nylon wool columns for 2 h at 37°C to enrich for T cells, then eluted.[23] AC and T-enriched cells (TEC) were washed and resuspended in culture medium.

Preparation of EC suspensions

Epidermal sheets were removed from the forearm using a suction blister device under a negative pressure of − 150 mm Hg.[24] The sheets were incubated for 1 h at 37°C in 0·5% trypsin in phosphate-buffered saline and agitated occasionally. They were gently pipetted to disaggregate the cells after which autologous plasma (15%) was added and the EC washed, counted and resuspended in culture medium.

Preparation of Ag

A clinical isolate of HSV-1,[25] plaque-purifed and passaged *in vitro* in Vero cells, was inactivated by exposure to ultraviolet (UV) B radiation for 1 h (UVHSV) under two Philips TL 6 20 w/12 UV lamps (270–350 nm) (HSV titre before inactivation 10^8 plaque forming units [p.f.u.]/ml).

Lymphoproliferation assay

Three parallel assays were performed *in vitro* for each patient: (i) UVHSV (5×10^5 pfu) was added to 5×10^5 washed purified PBMC/ml in culture medium and Vero cells (3×10^2/ml) were added to a parallel control culture. The other two lymphoproliferation assays consisted of 5×10^5/ml TEC in culture medium to which varying numbers of the following stimulator cells were added; (ii) EC pre-incubated with UVHSV for 90 min (20 p.f.u./cell), with parallel control cultures to which EC pre-incubated with Vero cells were added (5×10^2 Vero cells for every 10^6 p.f.u. HSV added to stimulator cells); (iii) AC similarly pre-incubated with UVHSV and appropriate AC controls pre-incubated with Vero cells. All cultures were plated as quintuplicate 200 μl aliquots onto 96-well round-bottomed microtitre plates (Cel-Cult, Sterilin Ltd., Hounslow, U.K.), incubated for 5 days at 37°C in a humidified incubator with 5% CO_2 and then pulsed for 20 h with 0·75 μCi [^{3}H]-thymidine (Amersham, Little Chalfont, U.K.) per well. Cells were harvested onto glass-fibre filter-paper discs and counted for 1 min in toluene-based scintillant. Stimulation indices (SI) were calculated as: SI = (mean c.p.m. with HSV Ag)/(mean c.p.m. with control Ag).

Blocking of lymphoproliferation with monoclonal antibodies

Purified EC or AC were pre-incubated for 30 min at 4°C with varying dilutions of the following four monoclonal antibodies (MAb) to MHC class II Ags which had been dialysed against culture medium before use: pan class II (DP, DQ, DR; DA6.231), DP (B7/21), DQ (BT3/4) (all gifts from Dr K.Guy) or DR (L243; ATCC). The cells were washed and incubated with UVHSV. They were then tested in parallel lymphoproliferation assays to those already described. On five occasions blocking experiments were undertaken in culture medium containing autologous plasma, heat inactivated for 1 h at 56°C before use. On four occasions cells were pre-incubated with MAb Leu 7 (Becton-Dickinson, Mountain View, CA, U.S.A.) or Dako T8 (Dakopatts, High Wycombe, U.K.) as control Abs.

Fluorescent flow cytometry

PBMC, TEC and AC were stained with murine MAbs to label the following cell surface Ags: CD1, CD3, CD4, CD8 (Dako T6, T3, T4 or T8, respectively; Dakopatts), CD11 (OKM1; Ortho Diagnostic Systems Ltd., High Wycombe, U.K.), as well as the anti-MHC class II Abs used for blocking experiments. EC were stained for CD1, CD11 and MHC class II Ag only. Cells were then washed and restained with sheep anti-mouse IgG F(ab′)$_2$ conjugated to FITC (Sigma). Cells were also stained with rabbit anti-human IgM (FITC conjugated F (ab′)$_2$ fraction; Dakopatts) to estimate B-cell numbers. Stained cells were analysed on a Coulter EPICS ‘C’ flow cytometer as described before.[22]

Enrichment for CD1$^+$ and DP$^+$ cells by cell sorting

Cell sorting of EC stained for CD1 and AC stained for DP surface Ags was performed using the EPICS on the basis of green fluorescence, acquired initially by gateing of the cell population in a two-parameter histogram, measuring the forward angle light scatter and 90° light scatter (flow rate 2000 cells/s, laser output 150 mW at 488 nm). Sorted cells were collected into sterile culture medium and aliquots re-analysed on the EPICS to measure CD1$^+$ or DP$^+$ cell enrichment. CD1- or DP-depleted and enriched cell populations were tested in the lymphoproliferation assay as described before but 10 samples for each cell population were compared instead of five. In one experiment CD1-enriched cells were fixed after the method of Karnovsky,[26] dehydrated with ethanol and embedded in Araldite. Ultra-thin sections were cut, stained with uranyl acetate and lead citrate and examined with a Philips M 301 electron microscope.

RESULTS

Analysis of blood and epidermal cell populations

PBMC, TEC and AC from 10 patients and EC from nine patients were stained for surface Ags, and cell subsets were measured by fluorescent flow cytometry. PBMC subsets were within normal limits for the local population (Table 1). TEC showed depletion of CD11 and MHC class II$^+$ cells and enrichment for CD3$^+$ and CD4$^+$ T cells. AC were largely depleted of T cells and enriched for MHC class II$^+$ and CD11$^+$ cells. Similar proportions of EC stained for pan MHC class II, CD1 and individual MHC class II Ags in contrast to the AC population, where many fewer cells stained for DP and DQ than DR.

Glass adherence and nylon wool adsorption functionally deplete the lymphoproliferative response of PBMC to HSV-1

PBMC were depleted of AC by glass adherence and non-adherent cells further enriched for T lymphocytes by nylon wool adsorption (TEC). The lymphoproliferative response of PBMC to UVHSV *in vitro* (median SI 8·9, interquartile range 5·1–13·2) was functionally depleted by

TABLE 1. The percentage of the PBMC, TEC and AC populations (10 patients) and EC population (nine patients) stained with different monoclonal Ab

Monoclonal antibody	% PBMC stained	% TEC stained	% AC stained	% EC stained
Dako3	58 (6)*	72 (8)	4 (2)	ND†
Dako4	45 (7)	54 (14)	4 (1)	ND
Dako8	24 (4)	22 (6)	4 (1)	ND
IgM	10 (2)	3 (2)	15 (8)	ND
DA6.231	14 (3)	2 (0·4)	68 (8)	5 (1)
L243	11 (3)	2 (0·3)	66 (5)	4 (1)
B7/21	6 (3)	2 (1)	16 (7)	3 (1)
BT3/4	6 (2)	1 (1)	16 (3)	3 (1)
Dako6	1 (0·5)	1 (0·4)	3 (1)	5 (1)
OKM1	13 (3)	2 (1)	66 (14)	1·6 (1)
2nd Ab Alone	1·1 (0·3)	1·2 (0·3)	1·5 (0·2)	1·8 (0·5)

* Standard deviation.

† ND, not done.

TABLE 2. A typical experiment showing depletion of the lymphoproliferative response of PBMC to UVHSV in the TEC population and its reconstitution by adding EC or AC pre-incubated with UVHSV to TEC

	Antigen			
Cell population	Nil	Con A	UVHSV	Vero
PBMC (5 × 10^5/ml)	596 ± 58*	39932 ± 1843 SI = 67	3229 ± 291 SI = 5·2	626 ± 75
TEC (5 × 10^5/ml)	207 ± 37	ND	239 ± 38 SI = 1·7	144 ± 15
TEC + EC (5 × 10^4/ml) pre-incubated with antigen	275 ± 24	ND	3471 ± 275 SI = 10·4	335 ± 172
TEC + AC (5 × 10^4/ml) pre-incubated with antigen	265 ± 25	ND	1570 ± 162 SI = 6·2	255 ± 24

* Counts per minute ± standard error.

these procedures so that TEC did not proliferate *in vitro* in the presence of UVHSV (median SI 1·4, interquartile range 0·96–1·9). A typical experimental result is shown in Table 2.

EC or AC pre-incubated with UVHSV restore lymphoproliferative responses of TEC

The lymphoproliferative response was restored by adding EC or AC pre-incubated with UVHSV to TEC as shown in Table 2. Variable numbers of EC and AC (between 3 × 10^3 and 3 × 10^5/ml) pre-incubated with UVHSV were added to 5 × 10^5/ml TEC in eight experiments and incubated. The SI produced varied with the number of stimulator cells added. A typical experimental result is shown in Figure 1. The peak SI produced by UVHSV pre-incubated EC was always higher than that produced by the same number of UVHSV pre-incubated AC,

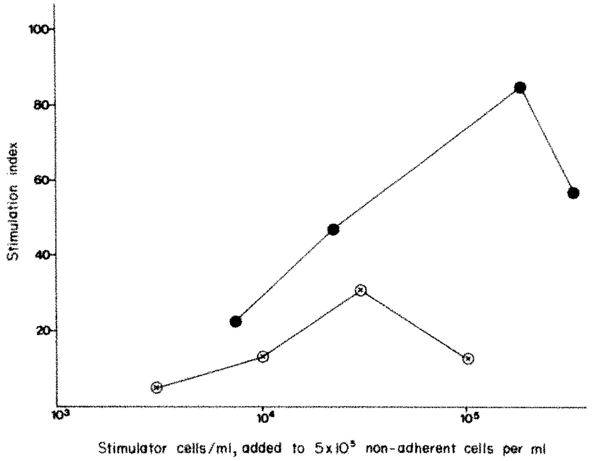

FIGURE 1. A typical experiment showing reconstitution of TEC lymphoproliferation to HSV by addition of EC (●) or AC (○) pre-incubated with UVHSV.

suggesting that EC are more efficient than AC at presenting HSV Ag to TEC on a per cell basis and per MHC class II$^+$ cell (5% EC, 68% AC MHC class II$^+$). Control cultures in which TEC were incubated with AC or EC alone or with EC or AC pre-incubated with Vero cells produced no lymphoproliferative responses (Table 2).

Abs to MHC class II Ags block lymphoproliferative responses to EC or AC pre-incubated with UVHSV

Blocking experiments were undertaken using anti-DP, -DQ, -DR or -pan MHC class II Abs prior to incubation of EC or AC with UVHSV. DA6.231, L243, B7/21 (12 experiments) and BT3/4 (eight experiments) produced concentration-dependent reductions in lymphoproliferative responses (SI) of 5×10^5/ml TEC induced by 5×10^4/ml EC or AC pre-incubated with UVHSV, and the pooled results are shown in Table 3. The highest concentration of each Ab produced a statistically significant reduction in $[^3H]$-thymidine incorporation in each case ($P < 0{\cdot}01$, two sample t-test). Pre-incubation of APC with either Leu 7 or Dako T8 as irrelevant control Abs produced no blocking of APC function (data not shown).

Depletion of CD1$^+$ cells from EC by cell sorting

In three experiments (Table 4) EC depleted of CD1$^+$ cells by cell sorting (5×10^4/ml) were compared with the same number of undepleted EC for their ability to present UVHSV to TEC (5×10^5/ml) in the lymphoproliferation assay. In each case the lymphoproliferative response was reduced below the background c.p.m. plus 3 SD by CD1 depletion and enhanced by enrichment for CD1$^+$ EC. In experiment 1, sufficient EC were available to count the approximate number of LC present in the CD1$^+$ enriched cell fraction by electron microscopy on the basis of typical dendritic morphology, folded nucleus and the presence of Birbeck

TABLE 3. Percentage blocking by MAb to MHC class II Ag of the ability of EC and AC to present UVHSV to TEC in a lymphoproliferation assay (anti-DP, -DR and -pan MHC class II Ab 12 patients; anti-DQ Ab eight patients)

Monoclonal antibody (ng/ml)		EC % Block	AC % Block
DA6.231	400	88 (4)*	69 (5)
	4	39 (13)	44 (3)
	0·4	8 (5)	2 (2)
L243	25	72 (4)	73 (4)
	1	41 (5)	36 (5)
	0·01	1 (0·5)	2 (1)
B7/21	50	79 (8)	75 (8)
	2	58 (4)	45 (4)
	0·2	22 (4)	30 (2)
	0·02	1 (0·6)	3 (1)
BT3/4	100	72 (6)	69 (8)
	10	36 (5)	40 (4)
	1	8 (2)	9 (4)

* ( ), Standard error.

TABLE 4. The effects of depleting CD1$^+$ cells from EC by cell sorting on their ability to present UVHSV in a lymphoproliferation assay

	SI and mean CPM ± SE* when TEC stimulated with					
	Whole EC		CD1-depleted EC		CD1-enriched EC	
Experiment	SI	CPM	SI	CPM	SI	CPM
1	5·3 (7%)†	1915 ± 194	2·8 (0·9%)	630 ± 71	13·1 (47·2%)	3721 ± 320
2	6·4 (3·7%)	1231 ± 141	0·8 (0·4%)	156 ± 12	18·9 (51·8%)	3972 ± 337
3	3·8 (2·7%)	592 ± 65	0·8 (0·3%)	93 ± 10	23·3 (55·9%)	2248 ± 101

* Counts per minute ± standard error.

† () Percentage of CD1$^+$ EC measured by fluorescent flow cytometry.

granules. This was estimated at 50% (20 cells counted) which corresponded well with the concentration of CD1$^+$ cells measured by flow cytometry (47%). In experiment 3, EC were stained for pan MHC class II Ag (DA6.231) as well as for CD1 Ag: similar numbers of EC stained for MHC class II Ag as for CD1 Ag (3·3%) and MHC class II$^+$ cells were depleted (to 0·93%) and enriched (to 52·6%) in parallel with CD1$^+$ cell numbers (Table 4).

Depletion of DP$^+$ cells from AC by cell sorting

To test the hypothesis that the APC function of AC is concentrated in a population of cells expressing DP, DQ and DR Ag, the effect of depleting and enriching for DP$^+$ cells, using cell sorting, on the APC function of AC for HSV was investigated. In four experiments, AC depleted of DP$^+$ cells were compared with undepleted AC for their ability to present UVHSV to TEC. A typical experimental result is shown in Table 5. In each experiment the SI was

TABLE 5. The effects of depleting DP$^+$ cells from AC on their APC function. AC were depleted of DP$^+$ cells by cell sorting and some were then pre-incubated with MAbs to MHC class II Ags. AC were then tested for their ability to present UVHSV in a lymphoproliferation assay

			Pre-incubated with MAb					
			DP (B7/21)		DQ (BT3/4)		DR (L243)	
Cell population	SI	c.p.m. ± SE*	% Stain	SI	% Stain	SI	% Stain	SI
PBMC	5	2636 ± 216	ND	ND	ND	ND	ND	ND
TEC	1	123 ± 8	ND	ND	ND	ND	ND	ND
AC	33	4399 ± 160	11	10	16	2	69	2
DP-depleted AC	19	2519 ± 157	1	16	7	1	34	3
DP-enriched AC	36	4585 ± 97	51	2	22	4	53	5

* Counts per min ± standard error.

markedly reduced by removal of DP$^+$ cells and enhanced by their enrichment; the reduction of [^{3}H]-thymidine incorporation by cultures depleted of DP$^+$ AC and the enhancement in DP-enriched cultures was also significant in each experiment ($P < 0{\cdot}01$, two sample *t*-test).

Blocking experiments were also undertaken with the whole AC, DP-depleted and DP-enriched AC populations (Table 5). In all three AC populations DR$^+$ cells predominated, although they were reduced in number in the DP-depleted population and increased in the DP-enriched population in three of the four experiments. Blocking with anti-DR Ab (L243) was high in all populations. Blocking with anti-DP Ab (B7/21) varied in parallel with the concentration of DP$^+$ AC present. The percentage of DQ$^+$ cells varied in parallel with the DP percentage and was highest in the DP-enriched populations. Anti-DQ Ab (BT3/4) blocked proliferation in all but the DP-depleted population in one experiment in which DQ$^+$ cells were also depleted to very low numbers (0·9%).

DISCUSSION

We have confirmed that EC and AC can act as APC for UVHSV Ag, as found by previous authors.[17-21] Presentation of HSV Ag by EC to responding lymphocytes is of great significance to the generation of the immune response to HSV infection since HSV causes predominantly epidermal lesions. Lymphoproliferative responses induced by EC pre-incubated with UVHSV were considerably higher than those induced by AC on a per cell basis, as previously demonstrated.[17-20,27] In addition notably fewer EC than AC stained for MHC class II surface Ag (Table 1).

The APC function of EC was concentrated in the CD1$^+$ population which was shown to contain LC by electron microscopy. The reduction of APC function by depleting the CD1$^+$ cells to 0·9% of the cell population agrees with previous studies,[28,29] which also found some residual APC activity at this level of CD1$^+$ cell depletion using different methods to deplete EC of LC and different models of APC function. In the other two experiments APC function was completely abrogated by further depletion of LC. APC function of EC for HSV was enhanced by enriching for CD1$^+$ cells as observed by other authors.[20] Since double labelling was not performed, it was not possible to measure how many of the MHC class II$^+$ EC were also CD1$^+$ but most MHC class II Ag bearing cells in normal skin are LC,[30] and in one experiment MHC class II$^+$ EC were depleted in parallel with CD1$^+$ LC. The numbers of CD1$^+$ cells were similar to those found by others,[3,20] as was the degree of purification of CD1$^+$ cells by cell sorting.[31] Approximately equal numbers of EC stained for each MHC class II Ag which is consistent with the observation that most LC stain for DP, DQ and DR Ag.[4] The small proportion of CD1$^+$ cells in PBMC was similar to that found by Dezutter–Dambuyant, Schmitt and Thivolet[32] who described these cells as having features suggestive of LC precursors.

Sontheimer[33] found that alloantigen presentation by EC was blocked by anti-DR Ab added during the first 48 h of culture and suggested that the Ab interfered with T-cell receptor–DR interaction. Other authors abolished the lymphoproliferative response to HSV Ag by treating macrophage-enriched PBMC and EC suspensions,[18] or by treating EC enriched for LC,[20] with anti-DR Ab and complement. The APC function of human EC for HSV was shown to be MHC class II restricted in the same way as monocyte APC function.[19,34]

It was interesting that MAbs to DP , DQ and DR blocked the APC function of both EC and AC for HSV in a concentration-dependent manner. The Ab preparations were dialysed against culture medium before use to eliminate any non-specific toxicity and heat-inactivated plasma was used in several parallel experiments to eliminate the possibility of complement fixation; no blocking occurred when irrelevant control Abs (Leu 7, T8) were used. The results of the blocking experiments suggests that cells bearing products of all 3 MHC class II loci may be involved in Ag presentation of HSV by EC and AC. The results of the DP$^+$ cell-sorting experiments (Table 5) are consistent with this.

DP and DQ Ag are usually found on a small proportion of AC relative to DR[35-37] as observed here (Table 1). Most of the APC function of human PBMC has been ascribed to circulating dendritic cells rather than monocytes. The dendritic cells are Fc receptor-negative, transiently adherent, poorly phagocytic and negative for monocyte specific surface Ag markers. They make up 2–5% of transiently adherent cells[37] and strongly express DP, DQ and DR surface Ag. Enriched circulating dendritic cell suspensions are reported to have similar APC function to EC suspensions for alloantigens[33] but are weaker APC than EC enriched for LC both for nickel- and tuberculin-purified protein derivative Ag[28,38] perhaps because of lower cell surface MHC class II Ag expression.[39] The blocking of APC function of EC or AC expressing more than one surface MHC class II marker (e.g. LC or circulating dendritic cells) by a MAb to one individual MHC class II Ag might be explained by steric hindrance interfering with the interaction between the sensitizing Ag or T-cell receptor and the other MHC class II Ags on the same APC.

Our study suggests that the cells responsible for APC function in both EC and AC suspensions express products of all three MHC class II loci and that in EC these are LC. An effective local immune response to HSV infection depends on CMI mechanisms, and presentation of HSV Ag by LC to responder lymphocytes is probably essential for this to occur.

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Malignant melanoma: aetiological importance of individual pigmentation and sun exposure

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SUMMARY

A case-control study of cutaneous malignant melanoma (CMM) was based on 523 incident cases and 505 age- and sex-matched controls selected from the general population. The purpose was to investigate the relative risk of developing CMM associated with different sun habits and indicators of pigmentation, such as skin type, eye colour and hair colour. Compared to people with black hair, blonde subjects had a relative risk of 74·4 (95% confidence interval, 45·8–120·8). Associations with skin type and eye colour were considerably weaker. Relative risks of about 1·5–2·5 were found for certain sun habits. The results suggest that in a population of Caucasian origin with a predominantly fair complexion, pigmentary status characterized by hair colour is a far more important aetiological factor than sun habits.

Cutaneous malignant melanoma (CMM) has shown a rapid increase in incidence in different Caucasian populations during the last 40 years.[1–3] In Sweden, there has been a more than fivefold increase among both men and women during the period 1958–1984 (Fig. 1). The mean increase in the age-standardized incidence rate 1960–1984 was 5·1% per year for women and 5·7% per year for men.[4] This is the most rapidly growing incidence of all malignancies in Sweden. In 1984, 587 women and 580 men with CMM were diagnosed. Since the incidence rate does not show any tendency to level out (Fig. 1), it is estimated that the total number of new cases in Sweden in 1988 will be approximately 1500.

Age-standardized mortality rates for 1961–1984 showed a mean increase of 2·4% per year for women and 3·5% per year for men. The mortality rate among men more than doubled during this period, while the increase among women was considerably lower (Fig. 2). The observed increase in new cases of malignant melanoma is mainly due to an increase in CMM, since the

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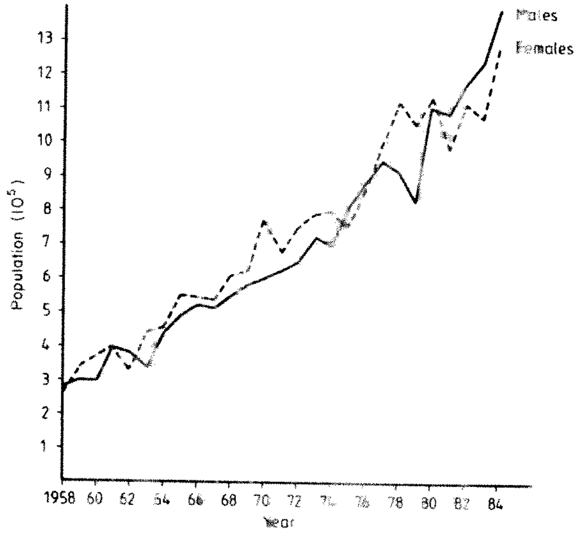

FIGURE 1. Trends in incidence rates of malignant melanoma of the skin 1958–1984 in Sweden by sex. Age standardized to the 1970 population census.

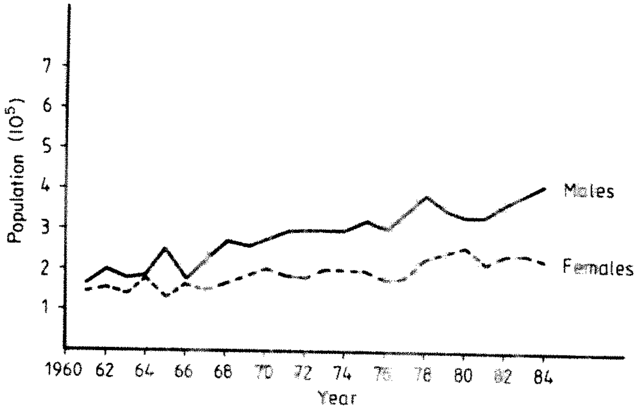

FIGURE 2. Trends in mortality rates of malignant melanoma of the skin 1958–1984 in Sweden by sex. Age standardized to the 1970 population census.

incidence of internal (ocular- and mucosa-derived) melanoma has remained essentially at the same level (1·4 cases per 100,000 population per year) during the same period of time.[5]

The reason for the rapid increase in the incidence of CMM is not clear, but altered sun habits have been suggested as a major cause.[5,6] However, the relation to sunlight exposure is not as straightforward for CMM as for actinic keratosis and squamous cell carcinoma of the skin.[7] The mean age at diagnosis is significantly lower for nodular (NM) and superficial spreading malignant melanoma (SSM) than it is for non-melanoma skin cancers and lentigo malignant melanoma.[8] This indicates that the cumulative dose of ultraviolet (UV) irradiation may be less important in NM and SSM than in the other malignant skin tumours. Furthermore, the location of NM and SSM is not predominantly on the sites most exposed to UV-irradiation.[9]

As early as 1896, Matas[10] reported on the low incidence of CMM among American Negroes. The life-time risk to develop CMM in the White population in the U.S.A. is 0·7%, while the corresponding figure for the Black population is 0·07%.[11] Black patients tend to have a different localization of their CMM compared to White patients, with a predisposition to develop acral melanomas in less pigmented skin. The same type of acral localization has been observed in Japanese and Chinese patients. Epidemiologic data from Israel also show different incidence rates among Jews of different ethnic origins, with the lowest figures among those of darker complexion.[12] The highest incidence rates of CMM are reported from countries at lower latitudes with a Caucasian population. However, in Europe the incidence figures are lower in Italy than those reported from the Scandinavian countries where the population is fair complexioned.[1,13] These observations point to the importance of the ethnic background of the population for the risk of developing CMM.

The purpose of this investigation was to study the relative risk of developing CMM associated with different sun habits and indicators of pigmentation (skin type, eye colour, hair colour).

METHODS

This case-control study originally included 525 patients referred from the Department of Oncology at the Karolinska Hospital in Stockholm, to the Department of Dermatology, during the period from February 1978 to December 1983. These cases constituted 63·8% (525/823) of all cases of CMM diagnosed in the population of Stockholm County during the corresponding period. The final examination and pathological classification of all tumours in this material, and for the whole County, has been carried out by Professor Bengt Lagerlöf at the Department of Pathology at the Karolinska Hospital (Table 1). All patients were asked to fill in a questionnaire while waiting for light testing at the Department of Dermatology.

A total of 525 controls were selected as an age and sex-matched sample from a population register covering Stockholm County. A questionnaire with an explanatory letter was mailed to each control. Identical questionnaires were used for cases and controls. All subjects included in the study were, at the time of the investigation, residing in Stockholm County.

The questionnaire included 10 questions related to sun habits, inquiring about the frequency of sunbathing, erythema following sunbathing, sunbathing vacations abroad, the use of 'sun protection agents', exposure in solariums, as well as experience of outdoor work and of living abroad. There were also three questions designed to characterize the pigmentary phenotype, inquiring about skin type according to Fitzpatrick,[14] eye colour and original hair colour.

TABLE 1. Classification of CMM in different tumour types in the county of Stockholm, related to those included in this study (Cases) during the period February 1978–December 1983

	SSM	NM	LMM	UMM	Total
Stockholm	581 (70·6%)	121 (14·7%)	16 (1·9%)	105 (12·8%)	823
Cases	363 (69·4%)	65 (12·5%)	8 (1·5%)	87 (16·6%)	523

SSM, superficial spreading melanoma; NM, nodular melanoma; LMM, lentigo maligna melanoma; UMM, unclassified malignant melanoma.

Relative risks were estimated using the Mantel-Haenszel procedure, and accompanied by 95% test-based confidence intervals based on the Mantel–Haenszel test. The data were stratified by age and sex. In the analysis of the associations between CMM and sun habits, the data were also stratified by indicators of pigmentation, i.e. skin type, eye colour and hair colour.

RESULTS

The questionnaire was completed by 99·6% of the cases and 96·2% of the controls. Analyses were thus based on 523 cases (240 male, 283 female) and 505 controls (224 male, 281 female). Table 2 shows the relative risk of CMM related to different indicators of pigmentation. The strongest associations were found for hair colour. Compared to people with black hair, subjects with ash blonde or brown hair had a relative risk of 26·1 (95% confidence interval: 16·7–40·9) and subjects with blonde hair had a relative risk of 74·4 (45·8–120·8). The risk of developing CMM among people with red hair was similar to that among people with blonde hair.

The two other indicators of pigmentation—eye colour and skin type—were also associated with the risk of developing CMM (Table 2). However, these associations were not as strong as for hair colour. Compared to people with brown eyes, subjects with green eyes had a relative risk of 6·1 (3·5–10·9). Compared to people with skin type IV, subjects with skin type II and III both had a threefold increase in the risk of developing CMM. For subjects with skin type I the relative risk appeared to be somewhat lower, but this may be due to random variation since there was considerable overlapping between the confidence intervals (Table 2).

When studying the associations between sun habits and CMM, adjustment was made not only for age and sex but also for different indicators of pigmentation. It turned out, however, that eye colour and skin type did not confound the relative risks adjusted for age, sex and hair colour. Hence, the relative risks associated with sun habits, that are presented in Table 3 and below, were adjusted for age, sex and hair colour.

TABLE 2. The relative risk (RR) of developing CMM related to pigmentary status, stratified by sex and age

Pigmentary status	RR (95% confidence interval)
Skin type	
I	1·3 (0·6–2·7)
II	3·1 (2·0–5·0)
III	3·0 (2·1–4·4)
IV	1·0
Eye colour	
Green	6·1 (3·5–10·9)
Blue, grey-blue	2·6 (1·9–3·5)
Brown, mixture (mainly brown)	1·0
Hair colour	
Blonde	74·4 (45·8–120·8)
Red	69·5 (33·8–142·7)
Ash-blonde, brown	26·1 (16·7–40·9)
Black	1·0

TABLE 3. Relative risk (RR) of developing CMM related to different sun habits, stratified by age, sex and hair colour

Sun habit	RR (95% confidence interval)	
No. of sunbathes April–Sept. per year		
>30	1·8	(1·2–2·6)
20–30	1·6	(1·1–2·4)
<20	1·0	
Erythema after sunbathing		
Often, very often	1·7	(1·0–2·9)
Seldom	1·6	(1·1–2·3)
Never	1·0	
Sunbathing vacations abroad		
≥Once a year	2·4	(1·5–3·8)
Every second year	1·3	(0·9–1·8)
Never	1·0	
Employment of sun protection agents		
Often, very often	1·8	(1·2–2·7)
Seldom	1·4	(0·9–2·0)
Never	1·0	

A modest but statistically significant increase in the risk of developing CMM was found among subjects who usually sunbathe more than 20 times per year during the period April–September (Table 3). For subjects sunbathing more than 30 times per year, the relative risk was 1·4 after stratification for age and sex, and 1·8 when stratification was also made for hair colour. A similar increase in the risk was found among subjects who tend to stay in the sun until an erythema develops. People going for sunbathing vacations abroad at least once a year had a relative risk of 2·4 (1·5–3·8). Some excess risk was also found among people using 'sun-protection agents' (Table 3). People who had lived in countries around the Mediterranean Sea or in countries with a subtropical or tropical climate for more than 1 year during the past 10 years had a relative risk of 1·9 (1·0–3·6). No increase in the risk of developing CMM was associated with sunbathing during winter vacations at high altitudes or frequent exposures in solariums. A tendency to reduced risk of developing CMM was seen among outdoor workers, who had a relative risk of 0·6 (0·4–1·0) after adjustment for age, sex and hair colour.

DISCUSSION

The age distribution and the distribution of tumour types (Table 1) among the cases in this study were similar to that of all cases of CMM diagnosed in Stockholm County during the corresponding time period and that observed in most of the previously reported studies of CMM.[15] For the validity of the present case-control study, however, the crucial question is whether the cases included in the study were representative of all cases of CMM occurring in the Stockholm population during the observation period with regard to sun habits and indicators of pigmentation. All patients examined at the Department of Oncology during the observation period were referred to the Department of Dermatology and therefore it is not likely that the cases included in the present study were different from the other cases with regard to sun habits and pigmentation. Nevertheless, we cannot exclude the possibility of such differences as a potential source of bias in the present study.

Skin type, hair colour and eye colour were used in this investigation as indicators of pigmentary status. Of these factors, hair colour was found to be the strongest risk indicator for CMM. Relative risks associated with different skin types and eye colours were considerably lower. This is consistent with the findings of previous studies[16–18] but to the best of our knowledge relative risks of the same magnitude have not previously been associated with hair colour. Any misclassification with regard to hair colour is likely to be non-differential, i.e. of the same magnitude among cases and controls. Such misclassification would result in a diluting effect which gives an underestimation of the strength of the association, i.e. influences the relative risk towards unity.

Subjects with skin type I had a somewhat lower relative risk than subjects with skin types II and III. This finding may be due to random variation, since there was considerable overlapping between the confidence intervals. Furthermore, subjects with skin type I did not expose themselves as much to UV-irradiation as subjects with skin types II and III. The findings of the present study showed a lower risk of developing CMM among subjects with skin type IV than among subjects with other skin types, but did not provide evidence of differences between skin types I, II and III.

The relationship between UV-irradiation and CMM is based in part on studies showing inverse relations between geographical latitude and incidence of CMM.[19,20] Stockholm is situated on the eastern coast of Sweden 59°N and 18°E. The mean number of sunshine hours per year was somewhat lower during the period 1961–1975 (1906 h) than during the period 1931–1960 (1973 h), see Table 4. Thus, changes in the climate cannot explain the steep increase of CMM in Stockholm County and in Sweden (Fig. 1) during the past 30 years. Data on other environmental factors which could influence the incidence of CMM, such as the mean thickness of the ozone layer over Sweden, have not been available for evaluation.

TABLE 4. Mean number of sunshine hours in Stockholm (59°N, 18°E) during the period 1931–1975. Data obtained from the Stockholm branch of the Swedish Meteorological and Hydrological Institute (SMHI)

Year	Mean number of sunshine hours		Mean number of sunshine hours h/year
	April–September	October–March	
1931–1935	1454	381	1777
1936–1940	1513	368	1881
1941–1945	1513	412	1925
1946–1950	1469	433	1902
1951–1955	1458	375	1833
1956–1960	1427	454	1880
1961–1965	1340	512	1852
1966–1970	1505	389	1894
1971–1975	1514	458	1972

A factor that to some extent might explain an increase in the incidence of CMM is the increasing mean age of the population, since the incidence of melanoma increases with age. This is, however, taken into account in the comparisons presented in Figures 1 and 2.

It may be difficult to judge to what extent changes in diagnostic routines and criteria during the past 30 years have contributed to the apparent increase in the incidence of CMM. However, a large proportion of the melanomas diagnosed in Stockholm in recent years have been rechecked and the histopathological diagnoses confirmed in the vast majority of cases. It is more difficult to judge if differences in the diagnostic routines 10, 20 or 30 years ago led to an underdiagnosis of malignant melanoma in the past. This is, however, not likely to explain the increase in age-standardized mortality rates (Fig. 2). Available information suggests that there has been a real increase in CMM during the past 30 years.

Changes in the climate and in diagnostic routines and criteria cannot satisfactorily explain the steep increase in the reported incidence of CMM. Changes in sun habits may, however, have contributed to this increase. The findings of the present study suggest that frequent sunbathing and the habit of going for sunbathing vacations abroad increases the risk of developing CMM. Such habits have increased considerably during the past 30 years.

The strength of the association between sun habits and malignant melanoma may be underestimated if pigmentation is not taken into account. If subjects with more pigmentation increasingly tend to expose themselves to UV-irradiation, but have a lower risk of developing CMM owing to their pigmentation, confounding by pigmentation would tend to give an underestimation of the association between UV-irradiation and CMM. Such confounding may, however, be taken into account e.g. by stratification in the data analysis. In the present study, for example, people sunbathing more than 30 times during the summer season had a relative risk of 1·4 when the data were stratified for age and sex only. When hair colour was also taken into account by stratification in the data analysis, the strength of the association increased to a relative risk of 1·8.

The information on sunbathing in Table 3 and the significance of 20 or more sunbathing episodes per year does require some caution in view of the variation in intensity of UV-light, the enthusiasm of the individual and the amount of body area exposed.

In this study, as in other epidemiological studies,[21,22] a tendency to react with erythema after sunbathing seems to be associated with an increased risk of developing CMM; outdoor workers having a lower risk than indoor workers. Since the minimal erythema dose is increased in pigmented skin, the tendency to react with erythema is reduced. The data seem to support the view that a pigmentary melanin filter induced by chronic sun-exposure of the kind obtained in outdoor work decreases the risk of developing CMM. Erythema induced by UV-light in non-pigmented skin increases the risk. This leads to the apparent paradox that UV-irradiation can both reduce the risk of melanoma by activating the pigmentary system and induce DNA-lesions that might predispose to future disease. The complexity of the matter is further illustrated by a study indicating that 'sunburners', associated with a non-habitual exposure to UV-light, are more likely to develop irregular or atypical naevi than are habitual sunbathers exposed to relatively larger UV-doses.[23]

The present study does not offer any explanation for the finding that frequent users of 'sun-protection agents' seem to have a somewhat increased risk of developing CMM. One possible explanation could be that these agents allow individuals with poor tanning ability to spend more time in the sun than otherwise possible. Another possibility would be that some chemical agent in these products has the ability to affect the melanocyte DNA in such a way that the development of CMM is facilitated.[24] Sun-protection agents have proven to be effective in preventing non-melanoma skin cancer.[25] The observation in this study, that this might not be the case for CMM, calls for further investigation.

Results from other epidemiological studies show that the presence of a large number of naevi is associated with an increase in the risk of developing melanoma, especially SSM.[26,27] The present study, however, was based on questionnaire data and no examination of naevi was performed.

The results of this investigation show that when different possible aetiologic factors related to CMM are compared, pigmentary status is more important than sun habits. In a white population of predominantly fair complexion, this becomes most evident when pigmentary status is characterized by hair colour and not by eye colour or skin type.

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The initial effects on workload and outcome of a public education campaign on early diagnosis and treatment of malignant melanoma in Leicestershire

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SUMMARY

We report our experience of the initial effects of a publicity campaign directed at early presentation of malignant melanoma in Leicestershire. The campaign resulted in a dramatic increase in workload and, at the pigmented lesion clinic, the numbers of new patients rose from 12·3 to 54·5 per clinic. There was a large rise in the number of new melanomas presenting in Leicestershire: from 1·02 per week before the campaign to 1·88 per week in the immediate post-publicity period. This was statistically significant ($P<0{\cdot}001$). Although there was also an apparently encouraging rise in the percentage of thinner 'good prognosis' tumours, it was not possible to isolate this statistically from a pre-existing trend.

Malignant melanoma is a life-threatening cancer which is becoming more common in most countries, including the United Kingdom.[1,2] Because there is no satisfactory alternative to excision and because prognosis is clearly related to the depth of invasion much attention has been focused on attempts to ensure the tumours are removed as early as possible. In Scotland it was found that many patients did not seek advice about melanomas for considerable periods of time,[3] but a subsequent media and poster/leaflet publicity campaign in the West of Scotland, aimed at the general public encouraged an earlier presentation.[4]

Seven centres in the United Kingdom (including Leicester) have also undertaken such publicity exercises. With funding provided by the Cancer Research Campaign, pigmented lesion clinics were established, and in July 1987 local publicity campaigns were commenced in

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the areas served by the clinics. We report our initial experience of this publicity campaign, together with its effects on our workload and on the number and prognostic categories of the melanomas seen in Leicestershire.

METHODS

On 1 November 1986 a separate weekly 'pigmented lesion' clinic (PLC) was established in the dermatology department at The Leicester Royal Infirmary. All general practitioners in the area of the Leicestershire Family Practitioner Committee were informed of the clinic by post. They were told that its purpose was to examine (and treat where necessary) lesions suspected of being malignant melanoma and that referral to the clinic was by open access after initial consultation with the family doctor. They were asked to tell their patients to attend the next clinic and to send a letter of referral.

Patients were seen on a first-come, first-served basis, and were given a letter explaining that the clinic was screening for skin cancer. Details about their history and examination were recorded on special forms provided by the Cancer Research Campaign (CRC), but we also maintained separate hospital records. An additional biopsy session was also established to deal with biopsies specifically generated by the PLC.

The details of all melanomas removed and sent for histology in Leicestershire have been recorded on the central computer in the department of histopathology at the Leicester Royal Infirmary, regardless of where they were first seen or excised. One of us (AF) initially examined all these records and tabulated the cutaneous melanomas from 1 January 1982 to 31 October 1986 (when the PLC was established). The Breslow thickness[5] of all the tumours has been checked and all melanomas have been placed in one of three depth bands used previously to relate to good, moderate and poor prognosis: $< 1{\cdot}49$ mm; $1{\cdot}5$–$3{\cdot}49$ mm; $> 3{\cdot}5$ mm respectively. All subsequent primary cutaneous malignant melanomas removed in Leicestershire have been examined and recorded in the same way.

In addition to the above records 18 general practitioners in Leicestershire agreed to record all new patients presenting to them with pigmented lesions for a 13-week period covering the time before and after the planned publicity campaign.

In early July 1987 a public education campaign was conducted in Leicestershire which contained two main components.

Leaflets and posters

A set of posters and leaflets drawing attention to the need for vigilance and early treatment in malignant melanoma was produced by the CRC. These were distributed by the Department of Health Education at the Leicestershire Health Authority and placed in public places such as general practice waiting rooms, pharmacies and public libraries.

In the leaflets patients were encouraged to examine themselves, their partners and relatives, and to become 'molewatchers' for life. The leaflets also contained a detailed seven-point check-list of features which might signify malignant change.

Newspapers, radio and television

A press release was sent out by the CRC to the local media of Leicestershire. This included the daily newspaper and the local weeklies as well as two local radio stations and local television. News of the campaign appeared in the local newspaper and short interviews with one of us were shown on television in which the main message of the need for early diagnosis and treatment was stressed.

Because the PLC was started in November 1986 and the publicity campaign occurred in early July 1987, the time periods under observation were not all of the same length, but adjustments were made for this in the statistical analyses.

RESULTS

The publicity in July 1987 had significant effects both on workload and on the number of melanomas seen.

Workload

We have been able to analyse the results of the exercise on the workload of both our PLC and of the 18 local family doctors. Other clinicians have also told us that they noticed an increase in the number of pigmented lesions seen, but there are no quantitative data available.

The workload of the general practitioners taking part in the assessment exercise is shown in Figure 1. Before the publicity in July 1987 the 18 Leicestershire general practitioners saw an average of 0·6 patients per day presenting with pigmented lesions. In the post-publicity period this had risen to 1·4 lesions per day. This increase is statistically significant (Mann–Whitney U-test $P = 0{\cdot}028$).

The patient attendance in the first year of the PLC at The Leicester Royal Infirmary is shown in Figure 2. It can be seen that there was a dramatic increase in patient numbers immediately after the publicity, peaking in the first 2 weeks at over 90 new patients in each clinic. During the first 34 weeks of the clinic's operation, between 1 November 1986 and the publicity campaign,

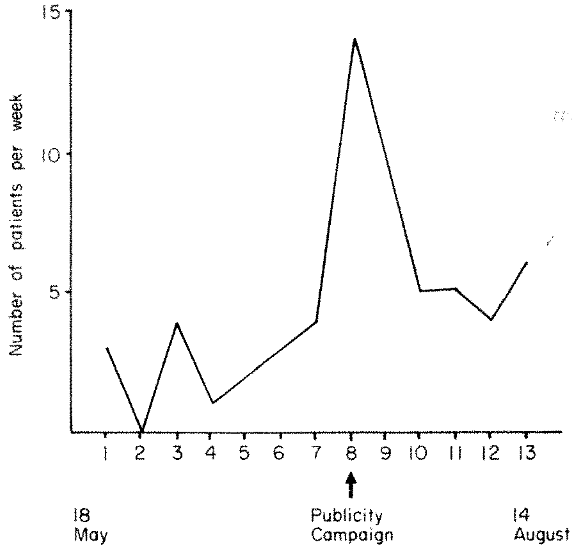

FIGURE 1. Numbers of new patients with pigmented lesions presenting to 18 Leicestershire general practitioners in the week beginning 18 May 1987 until the week beginning 14 August 1987. The week of publicity is marked with an arrow.

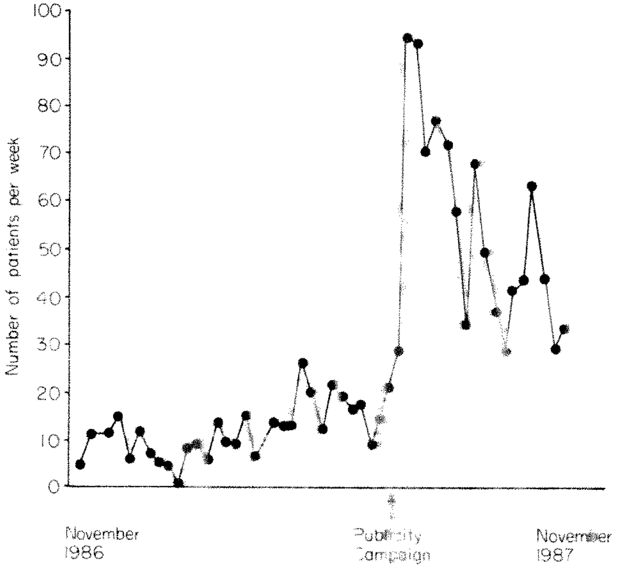

FIGURE 2. Numbers of new patients presenting each week to the PLC in Leicester from 1 November 1986 to 31 October 1987. The week of publicity is marked with an arrow.

we saw an average of 12·3 patients per clinic. In the following 17 weeks to the end of the first year, the average number of patients per clinic was 54·5. In total, 1344 new patients were seen in the first year of the PLC in addition to the routine work of the department. The numbers of patients seen in other dermatology clinics did not alter over this time as compared to the same 12-month period in 1985/6.

The PLC also generated an extra load on biopsy facilities. In the post-publicity period the number of biopsies in the separate weekly minor operations list, set up as part of the preparations for the publicity exercise, was an average of 8·8 per week.

Numbers of melanomas

The numbers of melanomas seen in the PLC also increased. We saw 15 in the first 34 weeks (0·44 per session), but 21 (1·24 per session) in the 17 weeks following publicity. The number of new patients seen in the PLC for every melanoma diagnosed rose from 28 in the pre-publicity period to 46 afterwards.

Of more importance than the experience in the PLC alone, however, are the figures for Leicestershire as a whole. Table 1 shows the total numbers of primary cutaneous melanomas recorded in Leicestershire during the relevant periods. These figures show a clear rise in the numbers of newly diagnosed melanomas in the post-publicity period. In the pre-publicity period of 34 weeks, we recorded 35 primary cutaneous melanomas (1·02 per week). In the 17 weeks immediately afterwards, we recorded 32 (1·88 per week). Statistical analysis was undertaken using an extension of the Poisson heterogeneity test (which takes into account the unequal time period lengths). The analysis was performed with the GLIM Package,[6] applying the χ^2 test. This demonstrated that the rise in numbers was significantly associated with the publicity in July 1987 ($P < 0{\cdot}001$).

TABLE 1. Numbers of melanomas seen in Leicestershire 1983–88

Period	Length (weeks)	Melanomas
1983	52	43
1984	52	24
1985	52	41
1 January to 31 October 1986*	44	41
1 November to 10 July 1987†	34	35
11 July 1987 to 31 October 1987‡	17	32
1 November 1987 to 30 April 1988§	26	35

* 1986 before PLC established.
† 1986/7 PLC established, before public campaign.
‡ 1987 post-publicity to end of first 12 months of PLC.
§ 1987/8 following 6 months.

TABLE 2. Prognostic thickness groups of melanomas seen in Leicestershire 1983–88 (figures are numbers with percentages in parentheses)

Period	0–1·49 mm	1·5–3·49 mm	> 3·5 mm
1983	13 (30)	22 (51)	8 (19)
1984	11 (42)	6 (25)	8 (33)
1985	19 (54)	10 (29)	6 (17)
1 January 1986 to 31 October 1986*	25 (61)	9 (22)	7 (17)
1 November 1986 to 10 July 1987*	21 (60)	5 (14)	9 (26)
11 July 1987 to 31 October 1987*	25 (78)	6 (19)	1 (3)
1 November 1987 to 30 April 1988*	22 (63)	7 (20)	6 (17)

* See Table 1 for relationship of time period to publicity.

The Breslow thickness of these tumours was determined to see whether there was any change associated with the publicity. Table 2 shows the prognostic groups into which the melanomas were placed. It can be seen that there has been a shift in the percentages of melanomas towards those lesions with a better prognosis from 1983 onwards. Immediately after the publicity, and up to the end of the first year of our PLC, there was a further increase in the percentage of good prognosis tumours, reaching 78%, with a fall in the numbers of melanomas in the worst prognostic group to 3%. In the following 6 months this trend returned towards the pre-publicity levels. However, these changes did not reach statistical significance. The numbers of melanomas in each category and in each time period were analysed using log-linear models, also fitted in the GLIM package.[6] The time from 1983 was used as a covariate in order to adjust for the underlying trend of improvement in prognosis. Such a trend was identified and, once removed, the July 1987 publicity was not significantly related to prognosis.

DISCUSSION

The role of sun exposure in the initiation of malignant melanoma is still unclear, but it is generally accepted to be a major factor.[7] Although the prevention of malignant melanoma by the alteration of harmful habits must be a long-term aim, it is important to improve its treatment and prognosis. The most important prognostic indicator for malignant melanoma is the depth of invasion by the tumour at primary excision[5] and early excision remains the most effective method of achieving a cure.

In the U.K., it appears that a rapid referral service alone is not enough; the establishment of a PLC in Southampton without a public education campaign had no effect on presenting melanomas.[8] The Glasgow group found that 84% of their melanoma cases in Scotland had delayed by more than 3 months before consulting their family doctor.[3] They concluded that a public education exercise was necessary and their initial results showed a rise in the number of newly diagnosed melanomas together with a significant rise in the proportion of thin, 'good prognosis' tumours.[4]

We have found a significant increase in the number of melanomas seen in the period after the publicity campaign. The increase of just under 100% in the first 17 weeks was even greater than that in the West of Scotland and our rate of one melanoma for every 46 new patients seen at the clinic is in keeping with that reported from Glasgow.[4] We also found a rise in the proportion of thin, 'good prognosis' lesions, but this was largely due to an absolute rise in the numbers of 'good prognosis' tumours presenting in the post-publicity period. It will be interesting to see whether overall numbers of melanomas, particularly in the thicker 'poor prognostic' categories, drop in succeeding years as a result of earlier diagnosis and treatment of thin lesions. The rise in thinner tumours was not significant as we already had an established trend towards thinner lesions, which may have been due to the previous publicity from Glasgow and the general increase in media coverage. The eventual return to pre-publicity levels suggests that the publicity campaign did have an effect.

The workload rose dramatically as a result of the campaign. Even with the extra support from the CRC, additional help and goodwill was required from the dermatology department. Clinicians in other departments were also aware of an increase in their melanoma-associated work. We also became aware of the need for better counselling facilities, since many patients were alarmed by the campaign and were shocked by the diagnosis of melanoma.

The CRC-funded unit nearest to us in Nottingham reported no significant increase in the proportion of thinner lesions in the previous year, and only a small increase in the numbers of melanomas seen.[9] We are unable to explain this difference.

The experience of the Glasgow group and that of ourselves suggest that public education of this kind is effective, although we will have to wait to see what effect, if any, there has been on 5-year survival rates. We emphasize that intensive local publicity campaigns as described here should only be undertaken if there is a well-organized structure to cope with the increased workload which will inevitably be generated.

ACKNOWLEDGMENTS

We are grateful to the Cancer Research Campaign who provided leaflets, posters and records materials and for financial support for JEO and SPL. We are also grateful to Miss Sally Nichols, Research Fellow and CRC Malignant Melanoma Education Co-ordinator, who collated the general practice data. We also particularly wish to thank our colleagues in the dermatology department at The Leicester Royal Infirmary, medical, nursing and clerical, and the 18 general practitioners who collected data. Finally, this work would not have been possible without the strong support and close co-operation of Messrs T.M.Milward and H.P.Henderson, Consultant Plastic Surgeons, The Leicester Royal Infirmary.

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Monoclonal antibody MAC 387 recognizes a myelomonocytic antigen shared by epithelial cells in inflammatory skin diseases

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SUMMARY

The monoclonal antibody MAC 387 recognizes an antigen expressed by human macrophages and granulocytes. Normal epidermis does not react with the antibody, but the inflamed epidermis may react. In this immunocytochemical study we have investigated the intracytoplasmic expression of the MAC 387 antigen in biopsies of a variety of skin disorders. In lichen planus the basal cells were usually negative, whilst suprabasal cells were positive. In the majority of other inflammatory dermatoses studied, there was positive staining of basal and suprabasal cells. A parallel frozen- and paraffin-section study of biopsies of cutaneous T-cell lymphomas and inflammatory conditions failed to demonstrate HLA Class II expression in the cytoplasm of keratinocytes. Expression of the MAC 387 antigen in the epidermis is directly associated with cell-mediated activity in the papillary dermis, but is not related to HLA Class II expression.

The monoclonal antibody (MAb) MAC 387 has been developed as a sensitive marker of macrophages and granulocytes in paraffin sections of formalin-fixed human tissues.[1,2] However, in biopsies of inflammatory skin disease, epidermal keratinocytes may also be labelled. In this study we have sought to characterize the extent of epidermal staining that may be expected. MHC molecules are induced in epidermal cells in similar circumstances, and we have also looked at MHC antigen expression in these biopsies.

METHODS

Biopsy material

An immunohistochemical study was performed on 35 routine formalin-fixed biopsy specimens

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TABLE 1. Results of immunocytochemical staining with MAC 387 in paraffin sections of various skin diseases

		Epidermal pattern			Dermal score		
Diagnosis	Total	0	1	2	0	1	2
Lupus erythematosus	5	1	1	3	1	2	2
Psoriasis	5	0	0	5	0	3	2
Lichen planus	5	1	4	0	0	1	4
Eczema	5	0	1	4	0	1	4
Bullous pemphigoid	5	0	3	2	0	0	5
Erythema multiforme	2	0	0	2	0	0	2
Sarcoidosis	3	3	0	0	0	3	0
Granuloma annulare	5	2	3	0	0	2	3

from different inflammatory skin diseases (Table 1). Paraffin blocks were obtained from the files of one pathology department and were all less than 3 years old. All histopathological diagnoses were confirmed by discussion with the two dermatologists who submitted the biopsies.

A further series of biopsies of various inflammatory skin conditions were chosen to investigate HLA Class II expression, using the monomorphic reagent WR18.[3] Paraffin blocks from 18 biopsies were used together with fresh frozen biopsies from a further series of patients with either cutaneous lymphoma or inflammatory skin disease.

Immunoenzyme staining

Paraffin sections were cut at 4 μm, dewaxed and treated with 0·5% H_2O_2 in methanol at room temperature for 30 min to abolish endogenous peroxidase activity. The primary antibody (Table 2) was incubated on the sections for 60 min at current dilution at room temperature, followed by 30-min incubations in peroxidase-conjugated rabbit anti-mouse immunoglobulins (Dako Ltd, High Wycombe, U.K.) at a dilution of 1:50, and peroxidase-conjugated swine anti-rabbit immunoglobulins (Dako Ltd), at a dilution of 1:300. Between each incubation there were three 10-min washes in Tris-buffered saline pH 7·6. Demonstration of peroxidase-active sites was achieved using diaminobenzidine substrate and nuclear detail by haematoxylin counterstaining.

TABLE 2. Antibodies used[4,5]

Antibody	Specificity	Sections	Dilution	Reference
MAC 387		Paraffin	1:5000	1,2
WR 18	m	Paraffin/Frozen	1:40	3
Tu 22	DQ[m]	Frozen	1:15	4
HLA/DR	DR	Frozen	1:40	(Dako)
B7/21	DP[m]	Frozen	1:25	5

[m], monomorphic.

Fresh tissue specimens were stored in liquid nitrogen and 10-μm sections were later cut using the slowest speed of the motor drive of a Leitz Kryostat 1720. The sections were fixed in dry acetone and stained using an indirect immunoperoxidase method. The primary antibody (Table 2) was incubated on the sections for 30 min, followed by three 2-min washes of Tris-buffered saline, a 30-min incubation with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins at a dilution of 1:50 (Dako Ltd) and three further 2-min washes with Tris-buffered saline. The colour reaction was developed with diaminobenzidine. Harris' haematoxylin was used as a nuclear counterstain and differentiated in 1% acid alcohol.

All sections were then blued, dehydrated, cleared and mounted in DPX. Serial sections were stained with haematoxylin and eosin to provide morphological controls, and were processed through the immunoperoxidase method without primary antibody to provide negative immunohistochemical controls.

Evaluation of staining

Epidermal staining in paraffin section was classified into one of three patterns. Pattern 0 represented negative staining, pattern 1 showed positive intracytoplasmic acanthocyte staining and negative basal cells, whilst pattern 2 showed positive intracytoplasmic staining of both acanthocytes and basal cells. Dermal staining was graded from 0 (no positive cells) to 2 (many positive cells). In frozen sections the presence or absence of intracellular and intercellular staining in the epidermis was noted.

RESULTS

Paraffin sections

Normal skin. No MAC 387 staining was seen in the epidermis of any of the specimens of normal skin examined.

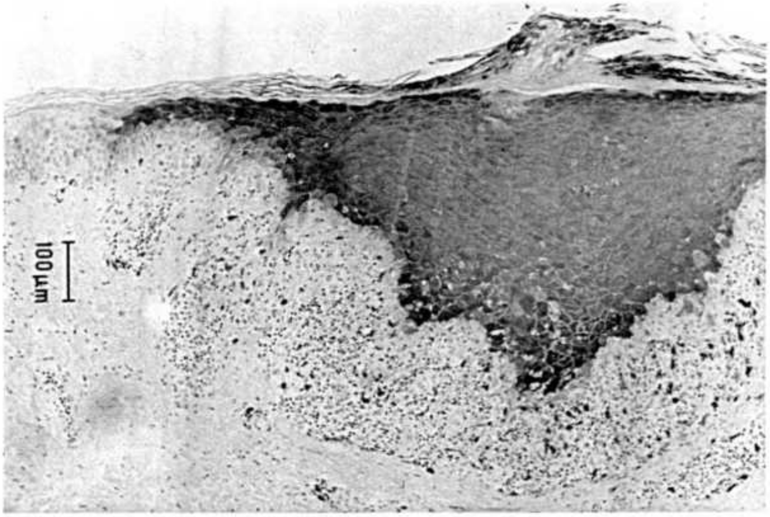

FIGURE 1. MAC 387. Pattern 1: positive intracytoplasmic keratinocyte staining with negative basal cells in a biopsy of lichen planus.

Diseased skin. Many of the biopsies showed cytoplasmic staining of epidermal cells (Figs 1–6). The results are summarized in Table 3. Dermal staining was confined to the cytoplasm of macrophages, Langerhans cells, granulocytes, and venular endothelial cells. Control sections incubated without primary antiserum were negative.

Lupus erythematosus. The epidermis was positively stained in four of the five biopsies and showed progressively stronger reactivity from the basal layer upwards. Acrosyringeal cells were negative. The hair follicle infundibulum was positive, but deeper structures in hair follicles were negative.

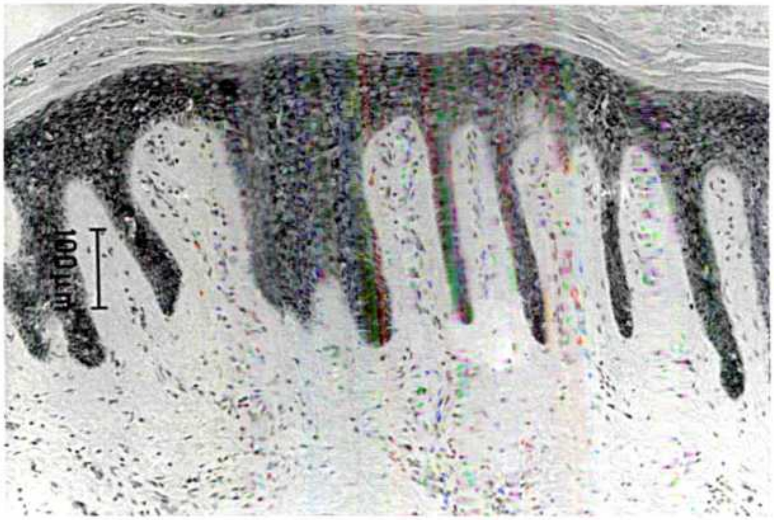

FIGURE 2. MAC 387. Pattern 2: Positive intracytoplasmic staining of basal cells and keratinocytes in a biopsy of psoriasis.

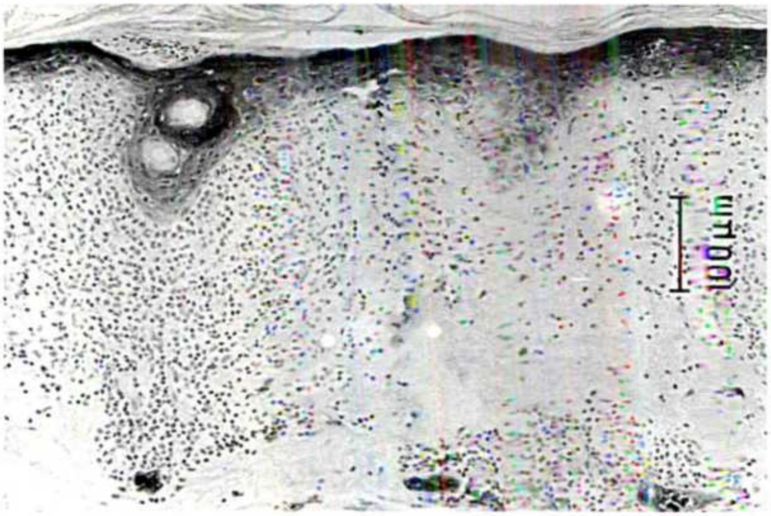

FIGURE 3. MAC 387. Pattern 2 with negative acrosyringium.

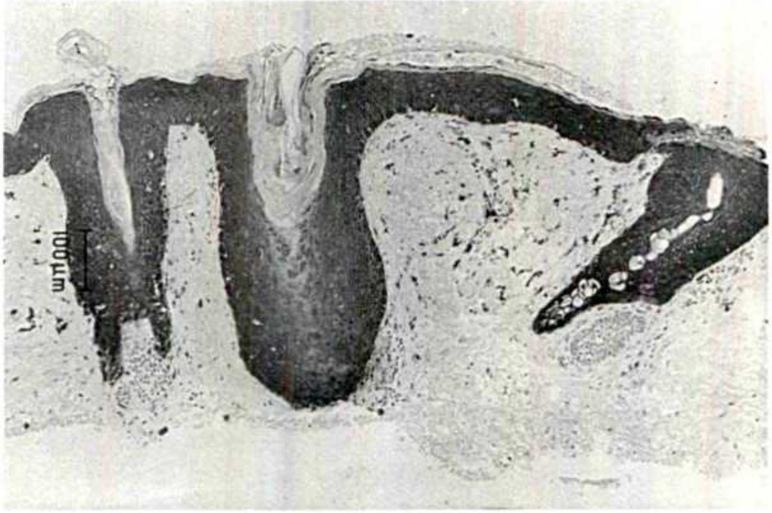

FIGURE 4. MAC 387. Pattern 2, showing positive epidermis and hair follicle infundibula with the negative staining of pilosebaceus units.

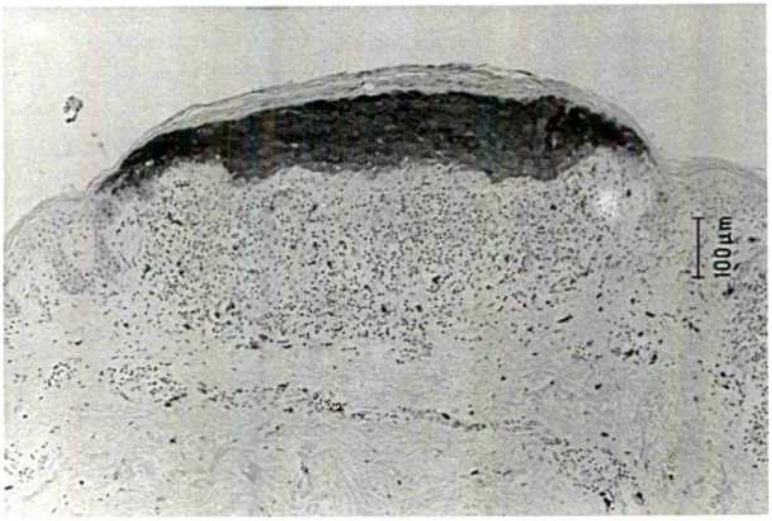

FIGURE 5. MAC 387. Biopsy of lichen planus showing sharp demarcation of positively staining lesional epidermis from surrounding negatively staining uninvolved epidermis.

Psoriasis. Epidermal cells were positive in all the biopsies and the reactivity was at about the same intensity throughout the epidermis including the basal cells. The acrosyringium was negative as were the sebaceous glands and subinfundibular hair follicle epithelium.

Lichen planus. Epidermal cells were positively stained within the lesions but not in the surrounding uninvolved areas. Basal cells in the epidermis showed weak or negative staining. Subinfundibular hair follicles were negative. The acrosyringium was not identified in the sections examined.

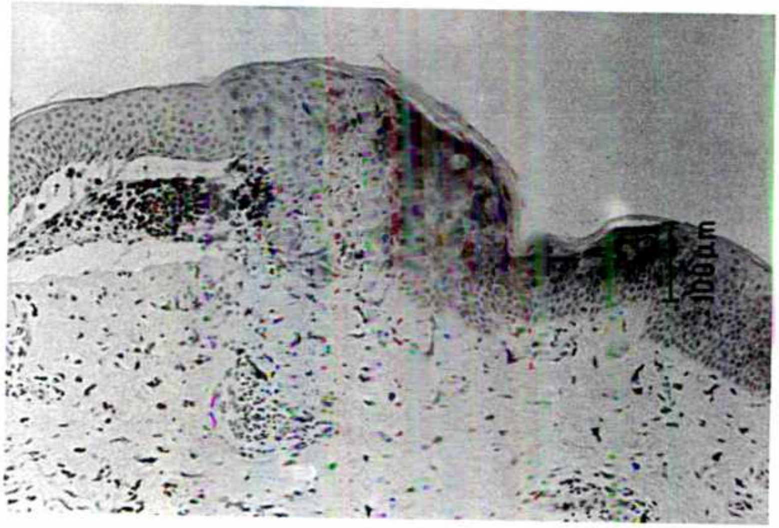

FIGURE 6. MAC 387. Positive staining of acanthocytes at the edge of a blister in a biopsy of bullous pemphigoid.

TABLE 3. Intraepidermal dendritic cell and intercellular staining in frozen sections of biopsies of cutaneous T-cell lymphoma and inflammatory dermatoses

	Lymphoma		Inflammatory	
Antibody	Negative	Positive	Negative	Positive
WR 18	0	13	0	8
Tu 22	10	3	7	1
HLA-DR	12	1	8	0
B7/21	5	0	5	1

Eczema. There was marked variation in the degree of staining in different biopsies which appeared to correspond to activity of the eczema. The basal cells were positive. Acrosyringial and subinfundibular hair follicles cells were negative.

Bullous pemphigoid. Positive staining was present in all of the biopsies, with less staining of the basal cells. The staining was mostly restricted to the epidermis at the edges of the blisters. Acrosyringial and subinfundibular hair follicle cells were negative. Cells in the central parts of sebaceous glands were positively stained.

Erythema multiforme. Positive staining was present in all of the biopsies, with less marked staining of the basal cells. Acrosyringial and subinfundibular hair follicle cells were negative. Cells in the central parts of sebaceous glands were positive.

Sarcoidosis. The epidermis was negative with the exception of cells in the acrosyringium and in hair follicles, and in sebaceous glands which showed weak, patchy positive staining.

Granuloma annulare. Three biopsies showed a patchy positive staining of the epidermal cells and two were negative.

MHC Class II: fixed tissue.

The additional 18 biopsies of a variety of inflammatory skin disorders that were stained with the monomorphic MHC Class II reagent WR18 all failed to show any epidermal staining, whilst parallel sections stained with MAC 387 were either strongly (15) or patchily (3) positive. Small numbers of lymphoid cells within the dermal infiltrates were stained by WR18.

MHC Class II: frozen sections

The results of staining for MHC class II antigens on frozen section are summarized in Table 3. None of these markers showed intracytoplasmic staining of keratinocytes. Positive reactions within the epidermis were restricted to dendritic cells and focal intercellular staining, probably representing staining of dendritic cell processes.

DISCUSSION

The monoclonal antibody MAC 387 is a sensitive marker of macrophages and granulocytes in routine paraffin sections.[1,2] In certain circumstances however, staining of epidermal cells is also seen in skin biopsies, a feature shared with the polyclonal precursor of MAC 387, the antibody S22. The L1 antigen is a highly immunogenic molecule which is biochemically distinct from HLA Class II molecules, but which is induced in similar circumstances.[6-10] The L1 antigen has three chains and it is likely that MAC 387 recognizes at least two of these.

Detailed immunocytochemical analysis employing sensitive double staining techniques strongly suggests that MAC 387 and L1 are present in identical cell populations.[4] Biochemical analysis of the L1 reactive material induced in keratinocytes showed that it was almost identical to that extracted from normal leucocytes, and a reaction of complete identity was obtained when both epithelial and granulocyte L1 were reacted against anti-L1 antisera by double immunodiffusion. These results are in accord with the findings of Gabrielson *et al.* with rabbit antibody to L1.[10]

MAC 387 expression in the epidermis is associated with lymphohistiocytic infiltration of the dermis, as we found in the wide range of inflammatory disorders studied, and the MAC 387 positive cells in the infiltrate corresponded to increasing degrees of staining in the epidermis. The demonstration that the MAC 387 antigen is induced in human epidermis in pathological states which parallel those which induce L1 is further evidence for immunological identity between the L1 antigen and the antigen recognized by MAC 387.

In the majority of biopsies with epidermal positivity, the acrosyringium and pilosebaceous units were negative, whereas it is known that these structures normally express MHC Class II antigen.[11] In sarcoidosis where histiocytic granulomas are present in the reticular dermis, but where the papillary dermis and epidermis are uninvolved, the only positivity was found in pilosebaceous units and sweat glands. This again suggests that positive staining in the epidermis is directly associated with cell-mediated activity in the underlying papillary dermis.

MHC molecules are induced in human leucocyte populations following activation and are also present in a variety of epithelia involved in inflammatory and neoplastic processes.[11-17] The application of WR18 in 18 fixed-tissue biopsies of inflammatory skin disorders shows that the conditions under which MAC 387 antigen is induced in inflammatory epithelia do not parallel those required for the induction of MHC Class II. This is confirmed by our data for the interaction of monoclonal antibodies to MHC Class II skin biopsies of frozen sections. Serial frozen sections to those studied in paraffin failed to demonstrate intracytoplasmic staining of MHC Class II antigens in epithelial cells with a range of monomorphic reagents directed to MHC sublocus products. Dendritic Langerhans cells were clearly demonstrated with associated intercellular staining. These findings emphasize that the intracytoplasmic expression of the MAC 387 antigen seen in paraffin sections is a parallel but different process to the expression of HLA Class II antigens within the epidermis.

The differential distribution of MAC 387 staining in different kinds of inflammatory skin disease should provide a basis for further work into the interactions between lymphoid cells and keratinocytes in inflammation and lymphomatous skin disease.

ADDENDUM IN PROOF

The L1 antigen is the same as the calcium binding proteins, the calgranulins A and B which are co-expressed during keratinocyte damage.[18]

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The permissive effect of sebum in seborrhoeic dermatitis: an explanation of the rash in neurological disorders

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SUMMARY

The hypothesis that sebum permits the growth of *Pityrosporum ovale*, and hence the development of seborrhoeic dermatitis, was tested by observing whether a reduction of sebum production by isotretinoin would improve the disorder. In 10 male patients with seborrhoeic dermatitis, treatment with isotretinoin for 6 weeks reduced the mean sebum excretion rate by 70% and improved the severity of the rash, but with a site difference in magnitude of response. It is concluded that the residual pool of sebum is important for the growth of *P. ovale* and that, within the physiological range, sebum has a permissive effect on the growth of this yeast. Variation in the pools of residual sebum explains a number of features of the disease such as site of involvement and greater prevalence in males than females. The pathological increase in the residual pool of sebum due to immobility explains the frequent occurrence of seborrhoeic dermatitis in patients with a variety of neurological disorders.

Recent studies have established the pityrosporal aetiology of seborrhoeic dermatitis and dandruff, its minor manifestation, and the condition responds rapidly to appropriate anti-yeast therapy.[1–3] Although *Pityrosporum ovale*, the causative yeast, is present in increased numbers in seborrhoeic dermatitis, the organism may also be found on the scalp of apparently normal subjects.[4] The factors influencing susceptibility to colonization by this yeast are therefore of interest.[1] The role of sebum was rejected by Kligman and Leyden[5] because of preliminary studies of scalp lipid, and by Burton and Pye[6] because the rate of sebum excretion is not increased in patients with seborrhoeic dermatitis. We found this unconvincing, since *P. ovale* is lipophilic and the disease occurs in areas of skin with the highest density of sebaceous glands, but not before they achieve adult activity. If sebum is important for growth of the organism which causes seborrhoeic dermatitis, reduction of sebum output should ameliorate the disease. To test this we used isotretinoin, a drug which greatly reduces sebum production.

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METHODS

Ten male patients aged 21–58 years (median 36 years) were studied. All had the typical red, scaling and crusting rash of seborrhoeic dermatitis affecting principally the paranasal, eyebrow or moustache areas. In addition to the facial rash, which was a requirement for entry into the trial, all patients were also found to have scalp involvement, and six had lesions of seborrhoeic dermatitis on the chest or back. Apart from non-medicated shampoo, which was used twice weekly, all topical treatment was stopped for at least 2 weeks before entry into the trial. Only one patient took any oral medication which was phenytoin for his epilepsy.

Isotretinoin (1 mg/kg) was given daily for 6 weeks. The patients were examined by the same two clinicians at the start of the trial, and at 2-week intervals for 10 weeks. At each examination forehead sebum excretion rate (SER) was measured gravimetrically,[7] and a clinical assessment made of the severity of seborrhoeic dermatitis involving the face, scalp, chest and back (0 none; 1 mild; 2 moderate; 3 severe). In addition, each subject made weekly assessments of the severity of rash on the face, and the degree of scalp scaling, using 100 mm visual analogue scales, the extremes of the scales being 'none' and 'severe'.

The paired t-test was used to analyse the significance of change in sebum excretion rate. All other tests of significance were performed using the Wilcoxon sign rank test.

RESULTS

Sebum excretion rate

All patients completed the study. There was a progressive decrease in SER during treatment with isotretinoin (Fig. 1), the mean SER (SE) being reduced from 1·21 (0.15) $\mu g/cm^2$ per min pretreatment to 0·36 (0·05) $\mu g/cm^2$ per min after 6 weeks ($P < 0·01$).

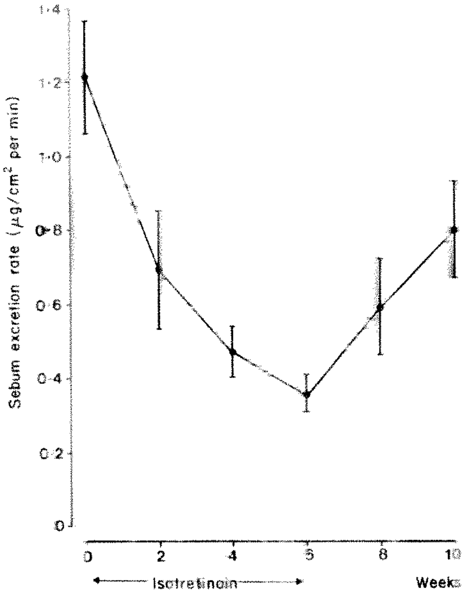

FIGURE 1. The change in mean (SE) sebum excretion rate during treatment with isotretinoin.

Clinical response

Unwanted effects. As expected, all patients developed cheilitis. Seven patients developed diffuse erythema and scaling of the face and seven developed an irritable, inflammatory, scaling rash on the hands, arms or shoulders.

Response of seborrhoeic dermatitis. Analysis of the clinical gradings made 2 and 4 weeks after stopping treatment, when the skin changes induced by isotretinoin had resolved, showed a small but significant improvement in severity of the facial rash compared with the pretreatment values ($P < 0{\cdot}05$), but only two patients were judged to be completely clear of the rash (clinical grade 0) (Fig. 2). The patients recorded a significant improvement in the severity of facial rash assessed using the visual analogue scales: median pretreatment score 57 mm; median score 2 weeks after stopping isotretinoin 10 mm ($P < 0{\cdot}05$). There was a decrease in the severity of scalp scaling

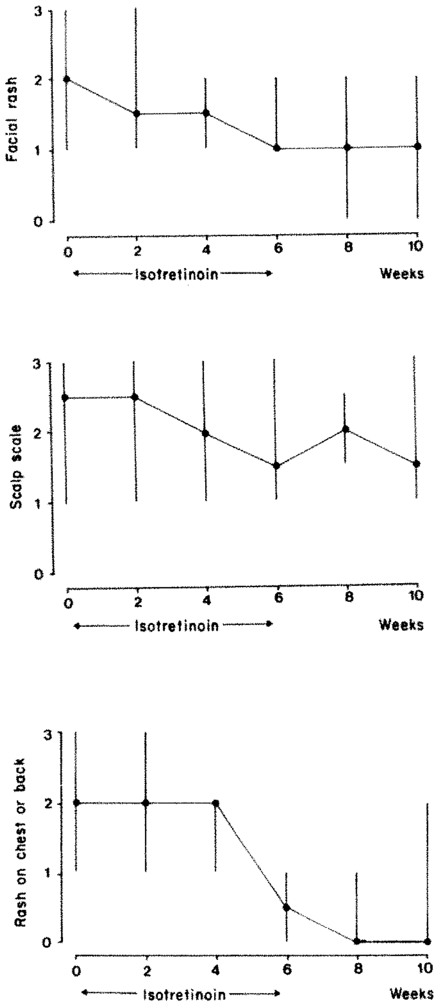

FIGURE 2. Clinical grading of severity of seborrhoeic dermatitis affecting the face, scalp and chest or back. Points are group medians and the vertical lines represent the range of values.

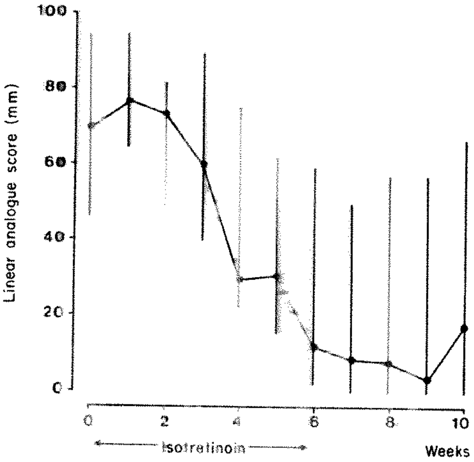

FIGURE 3. Change in severity of scalp seborrhoeic dermatitis assessed by the patients using visual analogue scales. Points are group medians and the vertical lines represent the interquartile range.

assessed clinically (Fig. 2) but this change was not significant and no patient's scalp was cleared of seborrhoeic dermatitis during treatment. However, the patients' own assessment, using visual analogue scales, showed a significant improvement after the 6 weeks treatment with isotretinoin ($P < 0{\cdot}01$) (Fig. 3). Six of the patients had annular, scaling lesions of seborrhoeic dermatitis on the chest or back, or an inflammatory folliculitis on the shoulders and back. The severity of rash at these sites, assessed clinically, improved significantly ($P < 0{\cdot}01$) during treatment with isotretinoin, the rash clearing completely in four (Fig. 2).

DISCUSSION

We have shown that during administration of isotretinoin to patients with seborrhoeic dermatitis, the rate of sebum excretion decreased and the rash improved. A reduction in SER occurred in all patients, and the mean reduction of 70% is similar to that found previously with the same dose of isotretinoin, as is the continued decrease over the 6 weeks of administration.[8] The therapeutic response was assessed only by a simple scoring method but both a response and a site variation were seen. The greatest response occurred on the chest and back and the least occurred on the scalp, where the clinically assessed change was not significant although, using a visual analogue scale, the patients' own assessment showed a significant improvement (Fig. 3). Although our study was not placebo-controlled, the site variation does not suggest a spontaneous or placebo response. We therefore conclude that improvement in the seborrhoeic dermatitis was due to the isotretinoin.

The rash which isotretinoin produces in its own right, and which occurred in our patients, may to some extent have disguised an improvement. For this reason we made clinical assessments 2 weeks after stopping the drug, when its epidermal effects had regressed but the decrease in sebum excretion was still apparent. The reduction in SER was progressive during the period of drug administration and anteceded improvement in the seborrhoeic dermatitis, as would be expected if the reduction in SER was the primary action of isotretinoin on this disease. To confirm this interpretation it will be necessary to show that other drugs which inhibit sebum production but do not have the other effects of isotretinoin, such as cyproterone acetate,[9] will similarly improve seborrhoeic dermatitis.

Because the maximum reduction in SER was only reached towards the end of the study, we cannot exclude the possibility that a longer period of administration of isotretinoin would have completely controlled the rash. Alternatively, the absence of a complete response as well as the site variation (see below) could be explained if the *in vivo* requirement of *P. ovale* for sebum is low. It would follow that the growth regulatory role of sebum for *P. ovale* is permissive within the physiological range, substrate dependence only becoming apparent when the changes are gross. This would reconcile our observation that seborrhoeic dermatitis is improved when SER is greatly decreased, with the observation of Burton and Pye[6] that patients with seborrhoeic dermatitis do not have an increased SER, and that variations in SER within the physiological range do not correlate with the severity of the condition. Furthermore, even if differences within the physiological range were not important within one sex this would not preclude an effect of physiological differences between the sexes. Thus, the difference in surface sebum between the sexes[6] could explain the greater prevalence of seborrhoeic dermatitis in men than women.[10] However, our study merely establishes the role of sebum and was not designed to test the effect of changes in sebum excretion within the physiological range, which we now believe should be reinvestigated.

Our conclusion as to the role of sebum also provides a novel and unifying explanation of the observation that seborrhoeic dermatitis is particularly common in patients with unrelated neurological disorders such as Parkinsonism,[11] cranial nerve palsy,[12] and major truncal paralyses.[13] We propose that a variety of neurological disturbances will provoke seborrhoeic dermatitis, and that this occurs because there is a pathological increase in surface sebum in these situations.[14,15] The apparent increase in SER in patients with various neurological disorders was first observed in Parkinsonism[14] where it was attributed to a neuroendocrine stimulation of sebum production.[16] There is no evidence of a neural control of sebaceous function,[17] and it was shown long ago that the techniques used to measure SER merely sample a large preformed pool of sebum in the stratum corneum.[18] It now seems likely that, as originally suggested in facial palsy,[19] an increased pooling of sebum due to immobility, and not an increase in sebum production, explains the apparent increase in SER in patients with neuromotor disorders.[17] Thus, it is changes in size of the static pool of already secreted sebum which permits growth of the yeast which causes seborrhoeic dermatitis. In this respect it is also important that hair is a major determinant of the size of the pool of sebum on the skin surface,[16] the greater maintainance of which would therefore explain why the scalp is usually the first site of occurrence of the disease as well as the site of least response to isotretinoin.

In conclusion, the effect of isotretinoin on seborrhoeic dermatitis suggests that sebum exerts a permissive effect on the growth of *P. ovale*, the causative organism. We suggest that it is the size of the performed pool of sebum and not its rate of production which is the determining factor, and that this explains the propensity to seborrhoeic dermatitis of patients with a variety of neuromotor syndromes, as well as certain features of the rash in otherwise normal individuals. We are not unaware of the irony in our painful achievement of this conclusion, when generations of patients and their doctors have known that washing improves dandruff.

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Topical propylene glycol and hyperosmolality

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SUMMARY

Topical propylene glycol is known to cause elevated serum osmolality in burns patients. Twelve adults with skin disease had quantities of propylene glycol applied to their skin to a maximum of 6·1 g/kg per 24 h for 5 days. Serum electrolyte, lactate and osmolality results remained unchanged during the study. These results suggest that topical propylene glycol may be used in adult dermatological practice without affecting serum osmolality.

The marked elevation in serum osmolality noted in burns patients has been shown in some cases to be caused by percutaneous absorption of propylene glycol (PG) present in topical silver sulphadiazine.[1-3] Though the presence of PG in a wide range of pharmaceutical and cosmetic agents has been encouraged by its apparent low systemic toxicity, this has been challenged recently by reports of significant side-effects from PG intoxication which include neurological problems, lactate acidosis, intravascular coagulation and hyperosmolality.[4-9]

There have been no reports of adverse metabolic effects from PG used in dermatological therapy, where it is particularly advocated for the treatment of ichthyotic skin disorders.[10,11] In Australia and New Zealand, 17% of dermatologists use PG in prescribed moisturizing cream.[12] Smaller quantities (usually less than 0·5 g/kg per day)[13] of cream are applied in dry skin treatment compared to burns therapy (up to 9 g/kg per day).[1] However, the concentration of PG in moisturizers is higher (up to 60%) compared to only 7% PG in silver sulphadiazine cream.

Osmolality can be directly measured[14] but can also be calculated using the formula:[3] calculated osmolality mmol/kg $= 1{\cdot}86\ (\mathrm{Na}^+) + \text{urea} + \text{glucose} + 9$. An excessive cutaneous absorption of topical propylene glycol could cause a significant gap to develop between measured (MO) and calculated osmolality (CO).[1,3] CO does not take into account all the solutes in the serum and is usually less than MO.

In this study, 30% and 60% topical PG cream was applied to adult patients with skin disease and MO was compared with CO to see whether hyperosmolality occurred.

METHODS

Preliminary toxicology study

Eight adult in-patients, with psoriasis or scaling disorders, had 30% PG in aqueous cream applied twice daily to their entire body for 1 or 5 days. Blood was collected, at entry or exit of the study, for urea, electrolytes, glucose and osmolality (MO). Osmolality (CO) was also calculated using the above formula.

Main study

Seven patients (three female, four male) with psoriasis (one patient was diabetic) had large amounts of 60% topical PG in aqueous cream applied for 5 days. Total-body occlusion-therapy was abandoned because of irritancy in some patients or incompatibility with adjunctive therapy. Blood was collected on entry, day 3 and at exit of the study for measurement of serum lactate, urea, electrolytes, glucose and osmolality levels. Blood for lactate studies was taken from flowing blood. Osmolality was also calculated. The amount of cream applied was measured and the patients weighed. The patients were all hospital in-patients and under observation for changes in general medical status.

RESULTS

Preliminary toxicology study

All blood tests and osmolality calculations were within normal range.

Main study

Three patients were eliminated because of inadequate measurements and skin irritation from topical propylene glycol. Two of the four evaluated patients had mild renal impairment reflected by urea levels slightly raised above normal. Most patients experienced severe desquamation during or after the study. No change in medical status was observed during the trial. A 70 kg adult would require, in normal practice, about 44 g of cream for total body coverage twice a day, giving an approximate dosage of 0·35 g/kg per 24 h.[13] In this study dosages varying from 1·5–6·1 g/kg per 24 h were applied. No significant change in measured serum osmolality was noted (normal range is 275–295 mmol/kg), nor did a significant osmolality gap develop between measured and calculated osmolality. Serum sodium, chloride and bicarbonate levels showed that an anionic gap had not developed. Lactate and BSL remained normal throughout the study (see Table 1).

TABLE 1. Calculated and measured osmolality results

Patient		1	2	3	4
Calculated osmolality (mmol/kg)	Entry	293	285	286	292
	Day 3	296	291	294	308
	Exit	284	292	296	302
Measured osmolality (mmol/kg)	Entry	302	301	294	285
	Day 3	311	322	304	315
	Exit	300	302	296	308
Quantity of 60% PG cream (g)		2350	650	1620	1715
Dosage g/kg per 24 h		6·1	1·5	5	5·9

DISCUSSION

Propylene glycol (PG) (1,2 propanediol) is a clear, colourless viscous alcohol that is miscible with water, and has many uses in industry, cosmetics and parenteral therapeutics. It is also used in the topical treatment of scaly skin disorders as a 20–60% aqueous or cream preparation and percutaneous absorption of PG is well documented.[1] About 45% of absorbed PG is excreted by the kidneys. The remainder is oxidized to lactate, pyruvate and acetate[15] and is a potential energy source. The half-life of PG in humans ranges between 10 and 30 h. The acceptable daily intake of oral PG for a 70 kg person is 1·75 g.[1] That same person applying a 60% PG moisturizing cream twice daily to the entire body would be exposed daily to approximately 26 g.[13]

Intoxication from PG is recognized and the reported side-effects include hyperosmolality, perception deafness, skin irritation, neurological problems including convulsions, lactate acidosis and acute intravascular coagulation.[4–8] Sublethal doses of PG in rats have haemolytic effects that are confirmed *in vitro* with PG solutions greater than 30%. Lethal doses do not seem to target any particular system or organ.[15] PG is less toxic than ethanol to human cells in culture[16] and has been noted to inhibit natural killer cell and neutrophil function *in vitro*.[17]

Hyperosmolality has been induced by PG in a number of clinical settings,[7] but particularly in intensive care unit patients during administration of nitroglycerin solutions that contain PG.[4,8] Hyperosmolality has also resulted from the percutaneous absorption of PG[1,2,3] in patients with burns. Silver sulphadiazine cream contains about 7% propylene glycol. Huge amounts of cream are applied in the treatment of burns patients, and one infant was reported as having 3400 g of cream applied over a 70 h period[1] resulting in a topical PG dose of 9 g/kg per 24 h. This patient's measured serum osmolality increased to peak at 420 mosmol/kg H_2O with an osmolality gap of 130 mosmol/kg due to the absorbed propylene glycol. Burns patients might be expected to have increased absorption because of an ineffective stratum corneum and vasodilation and may also have an inability to oxidize PG or excrete PG via the kidneys. Patients with toxic epidermal necrolysis and epidermolysis bullosa are sometimes treated with silver sulphadiazine cream, and dermatologists should be aware of this risk. Propylene glycol is more commonly used in the treatment of scaling skin disorders[10,11] where preparations containing up to 60% PG have been advocated and which may be used under polythene occlusion. Total-body occlusion therapy was abandoned in this study because of irritancy and incompatibility with other treatments. Occlusive therapy would be expected to increase percutaneous absorption.

In this study up to 6 g/kg per 24 h of topical PG were used for 5 days without significant changes in serum osmolality or lactate levels. Children have a larger surface area for weight so these results have not definitely established the safety of PG cream in treatment of paediatric skin disease. Patients with renal disease might also be at risk. Two of the patients in the final study had mild renal impairment but did not develop hyperosmolality. Most of the adults in this study had severe extensive skin disease and used up to 400 g of cream/24 h. They remained well throughout the study, with no clinical evidence of intoxication. While these studies provide only short-term data, in the typical practice of applying up to 44 g/24 h, topical PG is most unlikely to cause changes in serum osmolality or serum lactate in adult patients.

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Fresh versus cryopreserved cultured allografts for the treatment of chronic skin ulcers

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SUMMARY

Both fresh and cryopreserved cultured epithelial allografts were used to treat 20 patients with a total of 30 chronic ulcers. A profound stimulation of host epithelialization from the wound edges and epidermal appendages was observed. Our data suggest that allografts, prepared using a simple cryopreservation technique, retain their morphological and functional characteristics, and are as effective as fresh allografts in healing chronic ulceration.

Chronic non-healing ulcers of the extremities are a major health problem. In industrialized countries 0·5–1·5% of the population suffer from chronic leg ulcers[1,2] and in the U.K., over 500,000 patients currently receive treatment for leg ulcers.[3] In the U.S.A. in 1979, 128,000 patients were hospitalized primarily for stasis ulceration with an average length of stay of 11·4 days and which amounted to $300 million in health care costs.[4] In Leeds patients with leg ulcers accounted for 10% of all visits to follow-up clinics in the skin department and occupied 28% of the in-patient beds per year.[5] A treatment which is readily available and could reduce healing time, would be beneficial to both patient and the community. Previous studies have shown the effectiveness of cultured keratinocytes as allografts in healing chronic ulcers.[6–9] We have compared fresh and cryopreserved cultured epithelial allografts in the treatment of 30 chronic skin ulcers.

METHODS

Keratinocyte cultures

Cultured epidermal allografts were prepared from living HBsAG and HIV negative skin donors, using the 'feeder layer technique'.[10] At confluency the cultured sheets were detached from the Petri dishes after dispase treatment and mounted on Surfasoft.[11] They were either immediately used for 'fresh' grafting purposes, or cryopreserved and kept frozen until later use.

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TABLE I. Grafting of 30 chronic ulcers with cultured allogeneic keratinocytes

Underlying diseases	Grafting method	No. of patients	No. of ulcers	Mean duration (months)	Mean surface area (cm^2)	No. of grafts	Mean healing time (days)	No change
Venous stasis ulcers	Fresh	3	5	34	31	10	23	1
	Fresh + c.p.	6	10	7	20	17	29	2
	c.p.	5	8	15	26	10	26	1
Rheumatoid ulcers	Fresh	1	1	3	10	1	28	
	Fresh + c.p.	2	3	8	23	8	66	
Ecthyma gangrenosum	Fresh	1	1	3	320	2	84	
Erosive lichen planus	Fresh + c.p.	1	1	34 years	10	2		1
Pyoderma gangrenosum	Fresh	1	1	5 years	26	3	42	

c.p., cryopreserved (cultured epithelial grafts).

Cryopreservation and thawing procedure

The Surfasoft-backed sheets were submerged at room temperature in a cryoprotective medium containing 7·5% DMSO (dimethyl sulphoxide), EGF (epidermal growth factor) and 10% fetal calf serum using Petri dishes. The cultures were cooled by placing the dishes into a polystyrene box and placing the box into a mechanical refrigerator at −70°C up to 6 weeks before thawing and grafting. Temperature profiles of the cultures were recorded using a platinum resistance thermometer. The sensor was placed on the sheet and was attached to a digital microvolt meter (Keithly 197 Autoranging Microvolt DMM). Thawing occurred rapidly after incubating the cultures at 37°C in 10% CO_2 atmosphere and adding serum-free medium to the dishes. Post-thaw the cultures were characterized morphologically and per cent recovery of keratinocytes after trypsinization was tested with Trypan blue-dye exclusion and colony-forming efficiency.

Patients and grafting procedure

Twenty patients (six males, 14 females; mean age 67 years, age range 20–90 years) with 30 chronic ulcers, refractory to conventional or surgical methods of treatment, were treated with either: fresh allografts (8 ulcers); cryopreserved allografts (8 ulcers); or fresh followed by cryopreserved allografts (14 ulcers) (see Table 1). The ulcers averaged 33 cm^2 in surface area and 35 months in duration. They were grafted weekly or on alternate weeks until healing occurred.

The ulcer was prepared by debridement and Eusol compresses to produce a clean granulating wound. After application of the cultured sheets, tulle gras, a pad and an elastic bandage were applied. The patients were then confined to bed for 2 days. After 5 days the wound was inspected and subsequent dressings applied at 2-day intervals using a non-adherent type of dressing. Photographs were taken and the surface area of the ulcers calculated by weekly tracings of the edges on transparent polyethylene sheets and transferring these tracings onto graph paper.

RESULTS

In vitro assays

The cooling profile of cultures is shown in Figure 1. The initial cooling rate from room temperature to subzero was −1°C/min. The exothermic temperature plateau appeared at

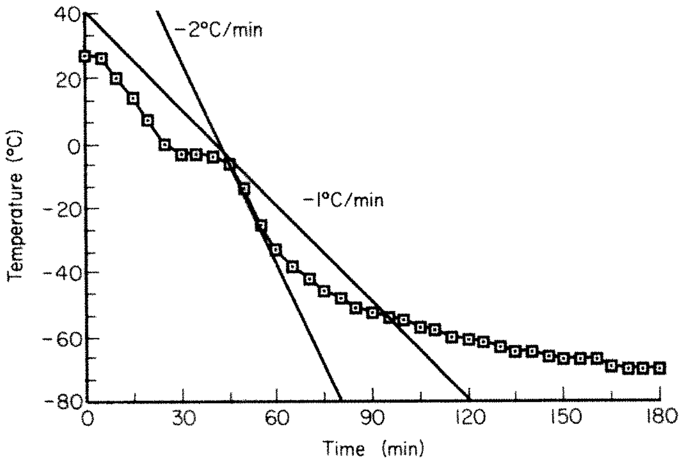

FIGURE 1. Uncontrolled-rate cooling profile of Surfasoft-backed cultured epithelium submerged in medium. Diagonal lines represent cooling rates of − 1 and − 2°C, respectively. Data points are plotted at 5 min time interval.

− 3°C, lasting 18 min. Maximum cooling rates, seen between − 10 and − 25°C, were not exceeding − 2°C/min, after which cooling slowed down progressively, reaching − 70°C within 3 h.

The post-thaw macroscopic and histological appearances were comparable to those of the fresh cultures. The post-thaw recovery rates of keratinocytes tested with Trypan blue exclusion and colony-forming efficiency were 62·8% (SD ± 16·8%) and approximately 50% of the index values, respectively, of the original cell lineage before cryopreservation.

Clinical results

In 75% of the patients (83% of the ulcers) there was complete healing with a mean healing time of 35 days. Venous ulcers healed twice as rapidly (27 days) as rheumatoid ulcers (57 days). No significant differences were noted in the mean healing time of venous ulcers treated with either fresh allografts (23 days), fresh plus cryopreserved allografts (29 days) and cryopreserved allografts (26 days).

The healing of ulcers occurred either as a partial or as a complete (in small ulcers) take of grafts in 40%, or migration of epithelium inwards from the edges of the wound in 43%. Often a translucent membrane structure was noted first, that became scaly and thick, and soon afterwards the skin appeared normal. Forty-three per cent of the ulcers healed within 4 weeks and 83% within 12 weeks. At 6 weeks a 70% reduction in area was seen in ulcers that did not achieve complete healing. Five ulcers (17%) in five patients showed no change. Two patients had diabetes mellitus and one patient had active and long-lasting erosive lichen planus affecting her feet.

Apart from early melting of islands of epithelium within some of the ulcers, no evidence of acute rejection or other adverse effects were observed after healing. One patient with chronic myeloid leukaemia developed an ecthyma gangrenosum after chemotherapy on his left thigh. Allografts eventually healed the ulcer and no rejection occurred before he died 3 months later from his leukaemia.

To date the longest period of follow-up after grafting is 28 months. Three of the venous ulcers had recurred by 6 months, and in two patients, this was due to failure of compression bandaging.

CASE HISTORIES

Case 1

A 82-year-old man had stasis ulceration around the medial and lateral malleoli that had failed to heal for 8 years (Fig. 2). Shortly before admission he developed an enlarging and painful

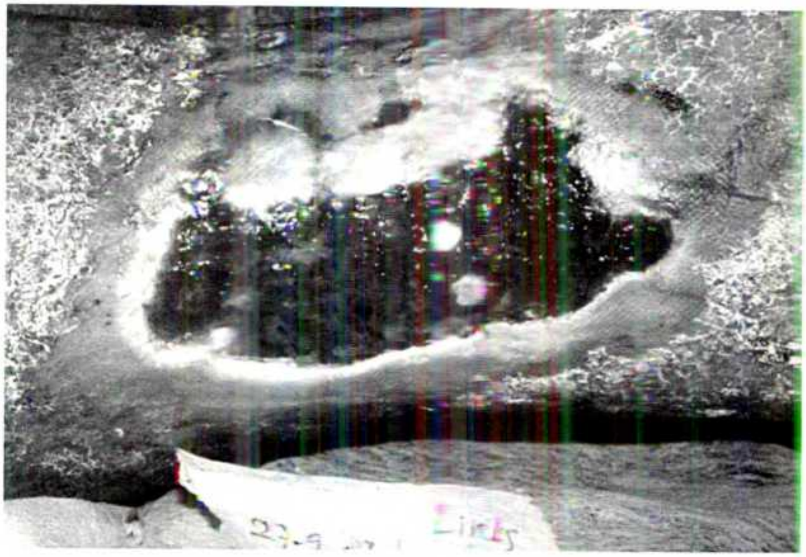

FIGURE 2. Case 1. Chronic end-stage stasis ulceration above the left lateral ankle.

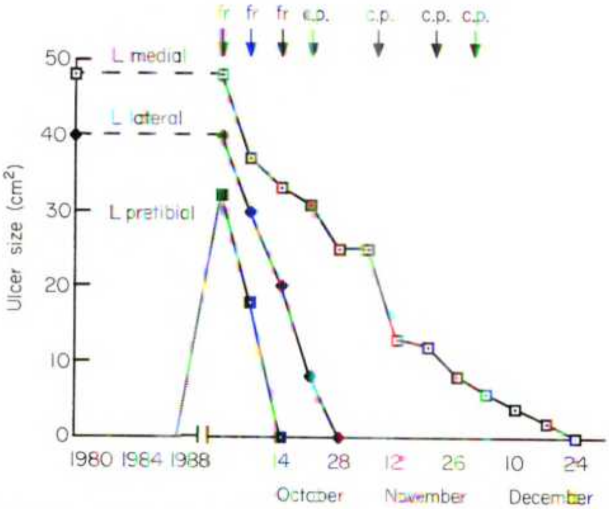

FIGURE 3. Case 1. Reduction in ulcer areas after treatment with fresh (fr) and then cryopreserved cultured allografts (c.p.).

pretibial skin ulcer. At regular intervals fresh and later cryopreserved allografts were applied to the ulcers (Fig. 3). Immediately afterwards the pretibial ulcer was free from pain and within 10 days new epithelium started to grow in from the wound edges (Fig. 4). The pretibial and lateral malleolar ulcers were healed at 16 and 25 days respectively. The larger ulcer around the medial malleolar was healed within 12 weeks. After each application of allografts to the ulcers there was a marked acceleration of healing and the cosmetic results were excellent (Fig. 5).

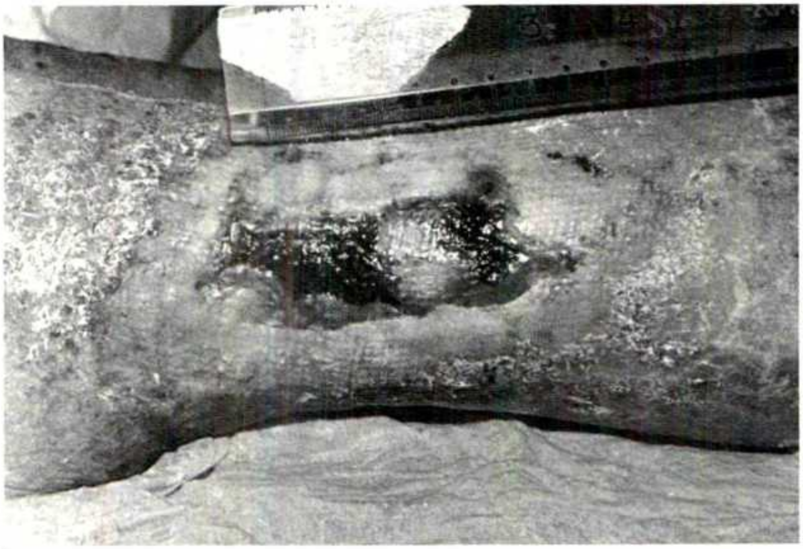

FIGURE 4. Case 1. Ten days after grafting a profound stimulation of epithelialization from the wound edges and epidermal appendages was observed.

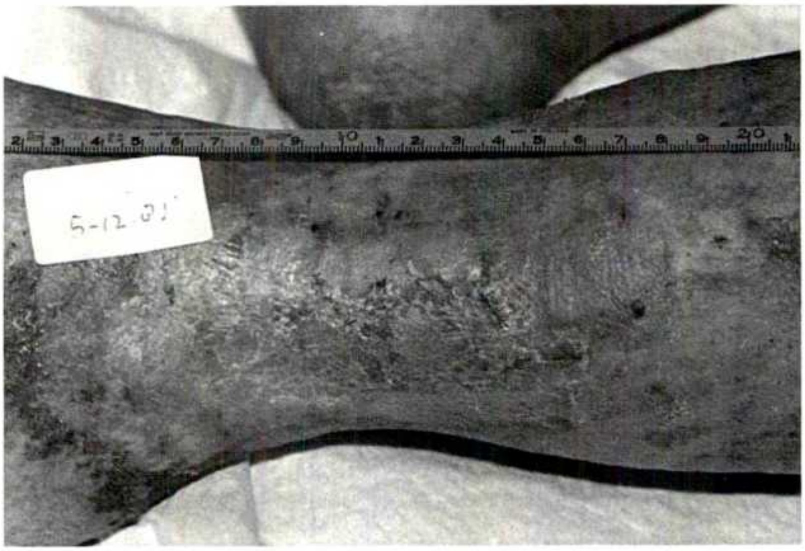

FIGURE 5. Case 1. Excellent cosmetic results after healing occurred.

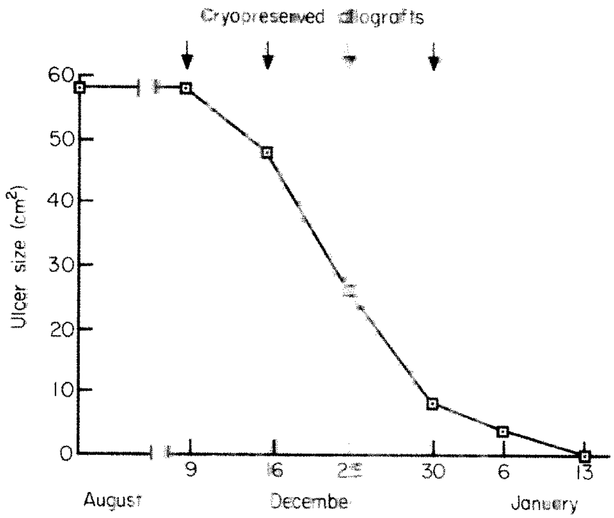

FIGURE 6. Case 2. Reduction in ulcer area after weekly applications of cryopreserved cultured allografts.

Case 2

A 71-year-old woman with a long history of recurrent venous ulceration developed rapidly enlarging bilateral ulcers with sloping edges, in a fibrotic area around the medial and lateral malleoli that extended over an area of 60 cm^2. After application of cryopreserved allografts (Fig. 6), the ulcers started to fill in rapidly with marked healthy granulation tissue and by 7 days the ulcers were covered with a translucent membranous structure. Four days later epithelial islands were observed in three ulcers that completely healed within 14 days. A larger ulcer epithelialized from the edges within 4 weeks.

DISCUSSION

Successful transplantation of cultured epithelial autografts was first reported in 1981[12] and this technique has since been employed in the treatment of extensive burns,[13-18] excised giant congenital naevi,[19] tattoos (I.M.Leigh, personal communication), degloving skin injuries,[20] junctional epidermolysis bullosa[21] and chronic skin ulcers.[22,23]

Following observations that cultured epithelial allografts were not clinically rejected in burn patients,[24] coupled with previous findings showing the loss of expression of class II antigens in cultured keratinocytes,[25] and supported by animal[26] as well as human studies[27,28] indicating the prolonged survival of cultured allografts, it was suggested that cultured allografts might be accepted permanently in unmatched recipients. Cultured epithelial allografts were successfully used for the treatment of extensive burns,[29] donor sites,[30] leg ulcers[6] and the eroded areas in recessive dystrophic epidermolysis bullosa.[7] Recent studies have shown that cultured allografts are not permanently accepted[31] but progressively replaced by the recipient's cells,[32] although graft survival is slightly prolonged.[31] The cultured allograft appears to act as a biological membrane, producing epidermal cell-derived factors. These factors seem to stimulate the migration and proliferation of keratinocytes from the wound edges and epidermal appendages and inhibit fibroblast-induced collagen contraction.[33]

In recent clinical studies it was reported that cultured allografts accelerate wound healing in patients with chronic ulcers,[6,8,9] the major effect being a stimulation of epithelialization from the edges of the ulcers. In one study[6] it appeared that epithelial islands in the centre of the ulcers survived temporarily and at least a transient take occurred. However after 1 week, no donor keratinocytes survived, indicating the striking migration and proliferation capacity of the stimulated host keratinocytes.

We have also observed a profound stimulation of host epithelialization from the wound edges and remnants of epidermal appendages, even in longlasting pyoderma gangrenosum and in therapy-resistent, chronic end-stage venous ulcers, as occurred in cases 1 and 2. After application of the cultured allografts most of the patients were immediately relieved of their pain, and the cultured grafts not only improved the granulation tissue, but promoted the formation of a complete basement membrane layer.[34]

We have developed a simple and effective cryopreservation technique using uncontrolled-rate cooling of cultured epithelium. Cooling and thawing are two critical periods for cell survival during cryopreservation. In order to promote the viability of frozen tissue, the usefulness of slow cooling and rapid warming procedures is generally recognized.[35,36] Prolonged exothermic temperature plateaux are particularly associated with damaging effects.[37]

Using our technique the cultures were not subjected to cooling rates in excess of -2°C/min. By cooling at that rate or less, Mazur[38] predicted that embryos ought not to freeze internally since they will have dehydrated to equilibrium before cooling to the ice-nucleation temperatures. Although prolonged exothermic temperature plateaux were present in our procedure, the recovery of keratinocytes after thawing was sufficiently high. These findings support the clinical results that cryopreserved allografts are equally effective as fresh allografts in healing chronic ulcers. More fundamental research on the specific cryobiological requirements of cultured epithelium is necessary. The reported advantage of controlled-rate cooling of viable human allografts[37] is, due to the multistrata structure of skin, not necessarily applicable to cultured epidermal sheets.

Our findings may have considerable importance in patient care, because cryopreserved allografts, as a biological 'off the shelf product', are readily available and can be easily transferred. The application of these allografts requires neither special skills, nor anaesthesia and donor site problems are avoided. No adverse effects of treatment have been observed.

Apart from early 'melting' of epithelial islands within some ulcers, no evidence of acute rejection was observed. Although we did not specifically study the cellular origin, it appeared that the cultured allografts in one case showed prolonged survival up to 3 months, presumably as a result of the profound state of immunosuppression in the patient.

Although we have followed our patients for up to 28 months, it is too early to assess the recurrence rate of ulcers after allografting, though there is no reason to expect that it would be any different from ulcers treated by other methods. Most important in the out-patient management of leg ulcers is supportive leg bandaging.[39]

Our data for both fresh and cryopreserved allografts suggests a strong stimulation of host epithelialization, with healing rates far better than reported for gauze and standard compression therapy.[40,41] We are currently conducting a comparative study of a hydrocolloid dressing and cryopreserved allografts in venous stasis ulceration.

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Topical retinoic acid for treatment of solar damage

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SUMMARY

Twenty patients with chronic solar damage of the skin were entered in a double-blind, within-patient trial to compare the effect of 0·05% tretinoin cream with a placebo applied once daily for 12 weeks. Sixteen completed the study. Clinical assessment of the individual signs of solar damage were recorded on separate visual analogue scales. After 12 weeks, there were significant improvements in fine wrinkling around the eyes, crease lines around the mouth and cheeks, wrinkling on the dorsum of the hands and yellow discoloration. Overall, 14 of the tretinoin-treated sides were judged to have improved compared to only two of the placebo-treated sides ($P=0{\cdot}011$). Measurement of skin thickness by pulsed A-scan ultrasound revealed that the sides treated with tretinoin were significantly thicker than the placebo-treated sides ($P=0{\cdot}019$). Skin biopsies taken before and after treatment showed an increase in mean epidermal thickness with tretinoin treatment ($P=0{\cdot}019$). The clinical signs of improvement persisted at the follow-up assessment performed 4 weeks after cessation of therapy.

Ageing of the skin in Caucasian subjects is characterized by wrinkling, loss of tone and turgor, a faint yellowish discoloration and the presence of telangiectasia.[1] Solar keratoses, lentigines and alterations of the skin surface add to the overall appearance of ageing. It is now generally acknowledged that these signs are primarily due to chronic solar damage from ultraviolet radiation (UVR) rather than the intrinsic ageing process, and the term 'photoageing' is used to describe them.[2] Recently it has been reported that many of these alterations are improved by the use of topical retinoic acid.[3,4] In this double-blind, within-patient trial we compared the effect of 0·05% tretinoin cream with a placebo in patients showing evidence of photoageing changes in the skin.

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METHODS

Patients

All the patients had attended the dermatology out-patient clinic with various ailments (10 basal cell carcinoma (BCC), five solar keratosis (SK), three BCC and SK, one sebaceous gland hyperplasia and 1 senile comedone). The only criterion for entry was the presence of one or more signs of photoageing affecting the forearms and face. These were: fine wrinkling around eyes, crease lines around mouth and cheeks, yellowish appearance to skin, telangiectasia over cheeks, periorbital comedones, wrinkling of skin on the backs of the hands and lesions of solar keratosis. Other cutaneous signs, e.g. dryness of the skin and irregular pigmentation were not considered to be due unequivocally to sun-damage and were not assessed. Ten male and 10 female patients with a mean age of 63 (age range 40–77 years) were entered into the study. All patients gave written, witnessed informed consent prior to entry into the study and the study received ethical approval from the Joint Ethics Committee of the South Glamorgan Area Health Authority and University of Wales College of Medicine.

Study design

The study was designed and conducted as a placebo-controlled, double-blind trial in which the active preparation and the placebo were randomly allocated to the right or left sides. Treatments were applied to the face and to the dorsum of the hands and forearms. The patients were given a tube of cream for each side and instructed to apply approximately 0·5 g of each to the extensor aspect of the allocated hand and forearm and the same amount to the ipsilateral half of the face. One tube contained 0·05% tretinoin cream (Retin-A cream, Ortho Pharmaceuticals) and the other contained the identical, amphiphilic vehicle cream with the substitution of a yellow colour in place of tretinoin. Both preparations were manufactured by Ortho Pharmaceuticals and were contained in identical, coded packaging. Monthly checks were made to ensure that the patients understood the need to avoid contamination of one side by the cream allocated to the other side and that the preparations were applied regularly. Tubes were inspected at each visit and weighed at the end of the treatment period to confirm compliance.

Patients were asked to apply the test creams once daily 2 h before bedtime and at least 30 min after washing. The treatment period lasted 12 weeks, during which time no facial cosmetics were to be used and exposure to strong sunlight was avoided. Patients were warned that irritation could result from treatment and that they should report any symptoms such as itching or burning.

Clinical assessments

Assessments by the same physician were made before treatment and at 4, 8 and 12 weeks, with a follow-up visit at 16 weeks. The individual signs of photoageing were scored on 0–10 visual analogue scales with separate scores for each side of the face and each hand. Patients were also evaluated for the signs and symptoms of skin irritation at each visit. Each side of the face and each forearm was assessed separately by asking the patient if there was any itching, stinging or burning and by inspecting for erythema or peeling and recording each of these parameters as either none, mild, moderate or severe.

At 4, 8 and 12 weeks of treatment the response to therapy of each side was rated by the physician as 'much improved', 'improved', 'slightly improved', 'no change' or 'worse'.

Objective clinical assessments

The skin thickness of the extensor aspects of the forearm were measured at the same sites on each arm at each visit using a pulsed A-scan ultrasound device (Cutech Ltd.).[5,6] Three measurements were made at the same point (at the midpoint of the junction of the upper and middle thirds of the extensor aspect of the forearm) and the mean value used.

A uniaxial extensometer (Cutech Ltd.)[7] was used to assess any change in skin 'stiffness' at the treated forearm sites. This device measures the forces generated during the forced movement of two metal tabs which have been fixed to the skin with a cyano-acrylate adhesive. The mean value of three readings taken from the middle third of the extensor surface of the forearm was used.

Histological assessments

Biopsies were taken at the central part of the proximal third of the extensor surface of each forearm using a 4-mm disposable trephine before therapy and after the 12 weeks' treatment period. The second specimen was taken from a separate site between 1 and 2 cm from the original biopsy. Specimens were fixed in buffered formol saline, embedded in paraffin wax, sectioned at 5 μm and stained with haematoxylin and eosin and by Halmi's trichrome method for staining connective tissue. The sections were examined by one investigator (RM) who did not know if specimens were taken before or after treatment or which treatment had been applied.

The histological sections were submitted to formal image analysis using the Optomax V image analysis (Analytical Measuring Systems). Image analysis of the haematoxylin and eosin-stained sections was performed to measure the mean epidermal thickness (MET). In this technique a high power field (× 40) is selected at random and the area of the epidermis is measured then divided by the length to give the average thickness of the epidermis. This was repeated 10 times for each specimen. The Halmi-stained sections were analysed in a similar way to measure the distance between the epidermis and the upper limit of the degenerate elastotic dermis, and the depth of the lower limit of the elastotic tissue.

In addition the following histological assessments were also made on each specimen and recorded on 0–10 visual analogue scales: epidermal dysplasia, dermal inflammatory infiltrate and dermal solar elastotic degeneration. These semi-quantitative techniques have been used previously for other similar assessments and have been found to be reproducible and to correlate with other, more objective methods.[8,9]

Statistical analysis

The changes in clinical signs between tretinoin-treated and vehicle-treated sides, the data from visual analogue scale assessments of the histological sections and the quantitative histological data were compared by the paired Student's t-test. The categorical data were compared by the Wilcoxon signed rank test or the χ^2 test.

RESULTS

Sixteen of the 20 patients completed the study. Three patients withdrew for reasons unrelated to the study. One complained of severe irritation from both tretinoin and vehicle creams and was unwilling to continue beyond the fourth week, although examination revealed only minimal erythema.

Clinical assessment at 4 weeks showed that two patients had slight overall improvement on the tretinoin-treated side (Table 1). After 8 weeks of treatment nine patients were impoved on the tretinoin-treated side ($P < 0.004$). At the end of 12 weeks of treatment, 14 of the tretinoin-

TABLE 1. Response to treatment

Treatment period (weeks)	Sites treated with tretinoin ($n = 16$)					Sites treated with placebo ($n = 16$)					Significance*
	Much improved	Improved	Slightly improved	No change	Worse	Much improved	Improved	Slightly improved	No change	Worse	
4	0	0	2	14	0	0	0	0	16	0	†
8	0	0	9	7	0	0	0	0	16	0	$P = 0.004$
12	0	4	10	2	0	0	1	1	14	0	$P = 0.011$

* Wilcoxon signed rank test.

† Difference too small to test.

TABLE 2. Results of treatment

Clinical signs	Treatment	Results: Before	After	Change with treatment	Difference between treatments	Significance
Mean visual analogue scale scores (0–10) (rounded values) for clinical signs						
Fine wrinkling around eyes	Vehicle	4·04	3·84	−0·21	−0·45	*P* = 0·039
	Tretinoin	3·76	3·10	−0·66		
Crease lines around mouth and cheeks	Vehicle	4·02	3·92	−0·11	−0·72	*P* = 0·008
	Tretinoin	4·09	3·27	−0·82		
Wrinkling on dorsum of hands	Vehicle	3·64	3·62	−0·03	−0·53	*P* = 0·013
	Tretinoin	3·75	3·20	−0·55		
Yellow discoloration	Vehicle	4·94	4·92	−0·02	−0·73	*P* = 0·007
	Tretinoin	5·02	4·27	−0·75		
Ultrasound measurements (mm)						
Dermal thickness	Vehicle	1·12	1·01	−0·10	0·13	*P* = 0·005
	Tretinoin	1·12	1·14	0·02		

treated sides were judged to have improved compared to only two of the vehicle-treated sides (*P* = 0·011).

Judged individually, fine wrinkling around the eyes, crease lines around the mouth and cheeks, wrinkling on the dorsum of the hands and yellow discoloration on the face all improved with tretinoin. Although wrinkles, crease lines and yellowing started to reduce after 4 weeks, the differences did not reach statistical significance until after 8 weeks. The differences between treatment with tretinoin and vehicle became more pronounced after 12 weeks of treatment (Table 2).

At the follow-up assessment it was found that the difference between the tretinoin-and vehicle-treated sites had become even greater (Table 3) despite the fact that no treatment was used during this period. The clinical assessments of the severity of periorbital comedones, telangiectasia and solar lentigo lesions showed no change with either the active treatment or vehicle.

The effect of treatment on solar keratoses was difficult to assess as only seven patients had lesions on both sides of the face or arms. Of these patients, two had an increase in the severity score with both tretinoin and vehicle, three decreased on both tretinoin and vehicle, but more on tretinoin, and two improved on the side treated with tretinoin and deteriorated on the side treated with vehicle alone.

Mild irritation occurred at tretinoin-treated sites in up to 94% of patients compared with 25% at vehicle-treated sites. However, this consisted of mild erythema in most cases and usually without any symptoms. There was complete disappearance of all signs of irritation at the follow-up visit.

TABLE 3. Follow-up assessments at week 36: visual analogue scale scores (0–10)

Clinical signs	Treatment	Score	Change from baseline	Difference between treatments	Significance
Fine wrinkling around eyes	Vehicle	3·87	−0·17	−0·66	*P* = 0·009
	Tretinoin	2·92	−0·83		
Crease lines around mouth and cheeks	Vehicle	4·04	0·02	−0·79	*P* = 0·005
	Tretinoin	3·32	−0·77		
Wrinkling on dorsum of hands	Vehicle	3·74	0·10	−0·78	*P* = 0·008
	Tretinoin	3·07	−0·68		
Yellow discoloration	Vehicle	5·02	0·08	−0·86	*P* = 0·002
	Tretinoin	4·24	−0·78		

Objective measurements

The skin thickness as measured by ultrasound showed a difference between tretinoin-treated sites and vehicle-treated sites, reaching a difference of 13% after 12 weeks of treatment ($P=0{\cdot}005$) (Table 2). This difference between treatments resolved when treatment was stopped.

There was no change in the skin stiffness measurements made with the extensometer at either tretinoin- or vehicle-treated sites.

Histometric results

There was a statistically significantly greater increase in MET with tretinoin than with vehicle ($P=0{\cdot}019$). There was no difference in the mean change in the depth or thickness of the elastotic dermis when tretinoin-treated sites and vehicle-treated sites were compared (Table 4).

TABLE 4. Histometric results

Measurement	Treatment	Mean depth (μm) Before treatment	Mean depth (μm) After treatment	Change with treatment	Difference	Significance
Mean epidermal thickness	Vehicle	53·5	59·8	6·3	14·0	*P* = 0·019
	Tretinoin	50·6	70·9	20·3		
Upper limit of elastosis	Vehicle	36·1	38·5	2·4	2·1	NS
	Tretinoin	45·3	49·8	4·5		
Lower limit of elastosis	Vehicle	41·9	44·1	21·6	−13·3	NS
	Tretinoin	43·3	44·1	8·3		

NS, not significant.

When the biopsy specimens were compared subjectively and scored on a visual analogue scale there was no significant difference between tretinoin or placebo in the degree of epidermal dysplasia, dermal elastotic degeneration or inflammation.

DISCUSSION

The serial clinical assessments of the skin on the face and hands reported here confirm earlier reports[3,4] that topical tretinoin improves the appearance of photoaged skin by reducing fine wrinkling, crease lines and yellow discoloration. The improvement in appearance of the skin was seen after 8 weeks of treatment and although the improvement was judged to be slight in most cases it persisted for at least 1 month after cessation of therapy.

Changes in the appearance of photoaged skin could be induced by several mechanisms. The presence of mild irritation with treatment in many patients raises the possibility that non-specific inflammation in the dermis with attendant swelling could be responsible. However, like Weiss *et al.*[4] we did not see any gross dermal oedema in the biopsy specimens and there were no histological signs of inflammation. If inflammation alone were responsible for the improvement in appearance then it would not be possible to explain the persistent improvement during the follow-up period when all physical signs of inflammation had resolved.

Previously we have shown that skin decreases in thickness with age, using an ultrasound technique.[6] The detection of an increase in thickness of tretinoin-treated compared to vehicle-treated skin may indicate either that the tretinoin-treated skin became thicker or the vehicle-treated sites became thinner. The most likely explanation is that there was a thickening of the dermis during treatment with tretinoin, similar to that observed in animal experiments.[10] However, we were unable to detect any difference in the histological appearance of collagen between treatments and this may have been because changes were taking place in the soluble components of the dermal connective tissue at the time of examination. The resolution of the skin thickness differences during the follow-up period raises the possibility that the change in the thickness was related to the irritation of the skin. There was a statistically significant increase in mean epidermal thickness during treatment with tretinoin (Table 4) which was similar to the findings in previous studies.[3,4] The visual analogue scale assessment of the effect on the degree of epidermal dysplasia of non-lesional exposed skin showed no difference between tretinoin and vehicle, but we were able to show that some patients with solar keratoses did improve at the tretinoin-treated sites.

The publication of the results of the first double-blind study was greeted with enthusiasm[11-13] and our results support these earlier findings that there is clinical improvement in the appearance of photoaged skin following treatment with tretinoin. Although we observed only slight improvement in appearance the changes do seem to persist for at least 1 month after cessation of therapy. However, there are still no data regarding the mechanism of action or the structural changes that take place within the dermal compartment. The reversibility and degree of change possible are also unknown and much more work is required before we can advocate the use of topical retinoic acid as a treatment for photoageing.

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Oral albendazole for the treatment of cutaneous larva migrans

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SUMMARY

Cutaneous larva migrans is becoming more common in the U.K. with the popularity of tropical countries as holiday destinations. We describe the increasing use of a new benzimidazole derivative, albendazole, which is very effective in the treatment of cutaneous larva migrans. In contrast to thiabendazole, it is virtually free from side-effects and should, we feel, become the treatment of choice for this condition.

Cutaneous larva migrans (CLM) is a distinctive eruption (Fig. 1) caused by infective larvae of various animal nematodes penetrating and subsequently wandering in the skin. The commonest larva involved is that of *Ankylostoma braziliense*, acquired from dogs, but several others have been implicated, including *Ankylostoma caninum*, *Uncinaria stenocephala* and *Bunostomum phlebotomus*.[1] Caucasians are most commonly infected while walking barefoot or lying unprotected on sandy beaches in tropical zones, where ova, shed with the host's faeces, have hatched into infective larvae able to penetrate human skin. Historically, cases presenting in the U.K. have returned from Central and South America or Africa. Recently, however, holiday destinations such as Thailand and Malaysia are responsible for an increasing number of cases.

CLM is usually self-limiting, most lesions resolving after 1–3 months (probably depending on the species of larva responsible[1]) as the human is a 'dead-end' host. Usually, however, the lesions are extremely itchy, and can be extensive so that treatment is often required. In recent years, a new oral benzimidazole derivative with potent antihelminth activity (albendazole, Smith Kline and French; Welwyn Garden City, Herts, U.K.) has been developed and used in the treatment of helminth infections such as hydatid disease.[2] In Central America, Africa, India and the Far East, albendazole has a product license for the treatment of intestinal helminth infections. In some of these areas, it has also been used, as a 3-day course, in the treatment of CLM.[3–5] This has proved to be very effective and has not been associated with any side-effects. In this report we describe our experience with albendazole in the treatment of CLM cases presenting in the U.K.

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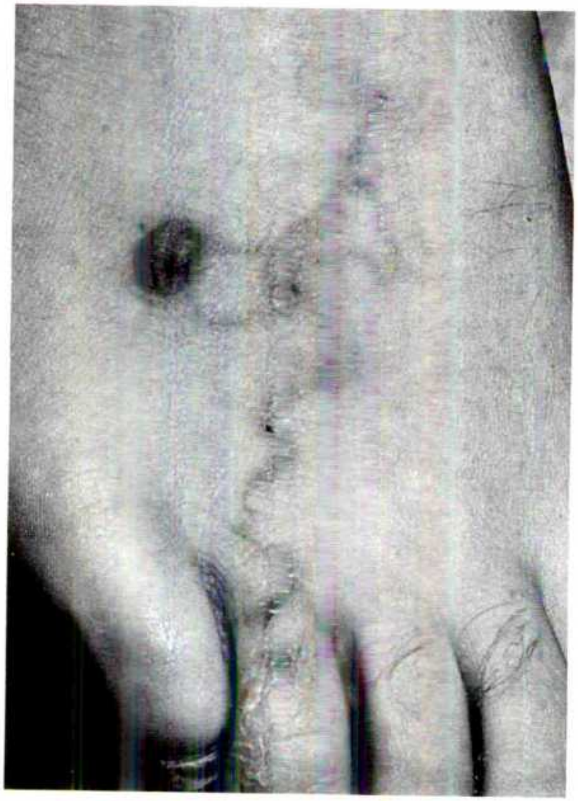

FIGURE 1. Typical wandering of cutaneous larva migrans. The circular lesion represents an unsuccessful attempt to arrest the progress of the larva with liquid nitrogen cryotherapy.

PATIENTS

Eight patients with typical CLM were treated with albendazole at a dosage of either 400 mg b.d. or 400 mg o.d. for 3 days; the reduction in dosage being suggested by the manufacturers during the study in the light of further patients treated world-wide. Individual patient details are outlined in Table 1.

All patients experienced rapid relief of pruritus (usually within 24 h) associated with the arrest of progression of the larval tracks. Most patients experienced complete resolution of lesions within 1–2 weeks. No patient experienced the severe side-effects frequently found with thiabendazole. Indeed, only one patient reported any side-effects at all, describing an uncontrollable temper on day 2 of treatment, just as he had experienced when being 'dried out' from alcoholism several years previously. No patient relapsed.

DISCUSSION

CLM is a benign, self-limiting eruption, but frequently causes extreme pruritus which requires treatment. To date, the therapies available have had marked side-effects, have been of doubtful value, or have been awkward to use. One method involves freezing with liquid nitrogen. This is rarely effective, however, as the larvae are ahead of the advancing track and can be missed, and

TABLE I.

Patient	Where contracted	Site	Dose (mg)	Symptom relief	Side-effects
AW	Jamaica	Foot	400 o.d.	24 h	Nil
SN	Jamaica	Foot	400 o.d.	8 h	Nil
CA	Thailand	Foot	400 b.d.	2 h	Nil
HW	Thailand	Calf	400 o.d.	3 h	Nil
LB	Thailand	Breasts	400 b.d.	24–48 h	Nil
VJ	West Indies	Toe	400 o.d.	24–48 h	Nil
AM	Antigua	Buttocks	400 o.d.	8 h	Temper
VC	Malaysia	Foot	400 o.d.	48 h	Nil

because larvae may not be killed by freezing (larvae survived 5 min exposure to −25°C in one study[6]). Other recognized treatments include thiabendazole given systemically (associated with possibly serious side-effects) or topically with or without occlusion (logistically difficult depending on the site).

In contrast, albendazole is rapidly effective[3–5] and, unlike thiabendazole, is well-tolerated. Although there have been isolated reports of abnormal liver function tests, neutropaenia and fever (returning rapidly to normal on stopping the drug), these have all been in patients treated for periods of 1–3 months and have not been seen in patients treated for just a few days. Its mode of action is not fully understood, but in common with other benzimidazoles, is thought to include: (i) inhibition of microtubule formation leading to impaired uptake of glucose by the helminth; (ii) inhibition of helminth specific fumarate reductase; (iii) inhibition of malate dehydrogenase.

Our series confirms the efficacy and safety of albendazole in Caucasian patients treated in the U.K. The recommended dose from this, previous studies, and data on file with the manufacturers to date, would seem to be 400 mg daily for 3 days, although in the future this may be reduced to a single 400 mg dose.[5] At present, albendazole does not have a product license for CLM in the U.K. but can be obtained on a named patient basis from the manufacturers.

ADDENDUM IN PROOF

Since this manuscript was accepted for publication a further report has confirmed the efficacy and safety of oral albendazole as a treatment for cutaneous larva migrans in Caucasion patients.[7]

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Aquagenic pruritus associated with the idiopathic hypereosinophilic syndrome

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SUMMARY

Patients with the idiopathic hypereosinophilic syndrome (HES) may develop associated skin disorders. We describe a patient who had xerosis since birth, but who first developed symptoms of aquagenic pruritus soon after he presented with HES. Photochemotherapy with psoralen and UVA treatment reduced his peripheral blood eosinophil count. The good response to treatment suggests that there was a close relationship between the dermatosis and the blood disorder.

CASE REPORT

A 61-year-old male patient was found to have marked blood eosinophilia ($24{\cdot}9 \times 10^9/l$) when undergoing investigation of longstanding osteoporosis of the spine. No cause for the eosinophilia was found, although he was an atopic subject. A bone marrow investigation showed hyperplasia of eosinophil precursors consistent with the diagnosis of the hypereosinophilic syndrome (HES). The leucocyte alkaline phosphatase score was normal and there was no Philadelphia chromosome. The eosinophilia persisted and a diagnosis of the idiopathic HES was made.

The patient also developed a haemorrhagic retinopathy which was initially thought to be due to hypereosinophilic thrombotic disease. It was, however, subsequently attributed to high myopia which is recognized as a cause of retinal degeneration. Severe degeneration can commonly lead to haemorrhage and retinal detachment.[1]

Shortly after presentation the patient developed symptoms of aquagenic pruritus. He complained of an intense discomfort of the skin, most marked 5 min after leaving his bath. Less marked pruritus occurred at other times. On examination he was found to have marked xerosis which had been present since birth. This had been asymptomatic till he developed eosinophilia, and his pruritus did not respond to treatment with emollients. His dry skin did not appear to be the cause of the aquagenic pruritus and it was concluded that the patient had developed aquagenic pruritus in association with the HES. He also had hand eczema. Clinical examination and investigation (including cardiac echograms) failed to show any evidence of other more usual manifestations of the HES, in particular, cardiac, pulmonary or neurological involvement.

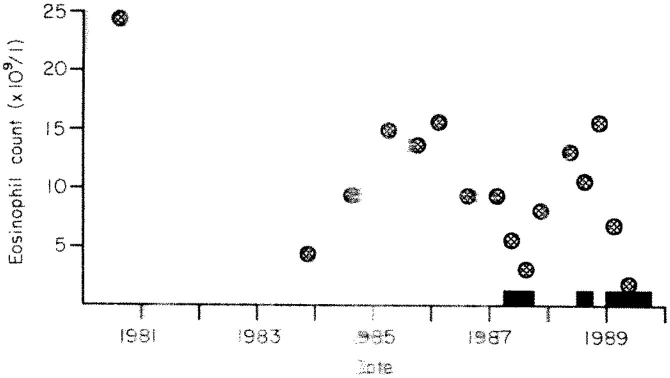

FIGURE 1. Progress of patient's eosinophil count with and without PUVA treatment. ■, PUVA twice weekly.

The patient received busulphan and prednisolone for a short period in 1981 following a mistaken diagnosis of 'chronic granulocytic leukaemia of eosinophilic type'. This resulted in a marked fall in his circulating eosinophil count and a considerable reduction in his pruritus. In 1984, the patient became intolerant of alcohol. Diarrhoea and incapacitating dizziness occurred after small quantities of alcohol. Alcohol intolerance is more frequently seen as a manifestation of Hodgkin's disease or carcinoma of the cervix[2] and has been previously reported in eosinophilic leukaemia.[3] The symptoms have subsequently persisted.

Investigations

A skin biopsy from the itchy xerotic, but otherwise normal skin of the thigh in 1987 showed only a mild perivascular inflammatory cell infiltrate with small numbers of eosinophils. Sections were also stained with the mouse monoclonal antibody EG2 (gift of Dr Tai, St George's Hospital) which stains secreted eosinophil cationic protein and eosinophil protein X and demonstrates intact eosinophil granules and extravasated eosinophil material. No significant specific staining was seen in the patient's sections.

Since cutaneous fibrinolytic activity has been claimed to be elevated in aquagenic pruritus[4] this was measured in a biopsy specimen from our patient's skin using a fibrin-plate method[5] but the results before and after water challenge were no different from the controls.

Progress

The patient's aquagenic pruritus became markedly less severe after chemotherapy produced a reduction in his circulating eosinophil count. The pruritus continued however, and PUVA-treatment was begun in 1987 and continued over 9 months. Although there was only a small reduction in symptoms, during this time the eosinophil count fell. The eosinophil count rose again when the PUVA was discontinued in 1988 and was further controlled when PUVA was reinstituted (Fig. 1).

DISCUSSION

Skin lesions are commonly present in patients with the HES, with an incidence of between 27%[6] and 53%.[7] The skin lesions are frequently non-specific, either red pruriginous papules over bony prominences or angioedema/urticaria.[7] There have been occasional reports of more extensive itchy papular eruptions.[8] Rarely, cutaneous lesions may be the only manifestations of the syndrome.[7] Aquagenic pruritus, has not, to our knowledge, been described in the HES. The histology of the affected skin in the HES shows a mixed inflammatory infiltrate with a high proportion of eosinophils. We failed to demonstrate increased numbers of eosinophils in our patient's skin possibly because it was biopsied at a time when his disease was in relative remission. The antibody EG2 was used in an attempt to pick up evidence of activation of eosinophils as it has been shown that eosinophil granule deposition may occur in dermatoses such as atopic eczema even in the absence of large numbers of eosinophils.[9] However, no specific staining was demonstrated.

Our patient also has congenital xerosis, probably associated with atopy. Xerosis may produce pruritus when associated with eczema, or in the elderly it may manifest as water-induced itching.[10] It is unlikely that xerosis was responsible for our patient's symptoms as the pruritus was of late onset, and was unresponsive to intensive treatment with emollients.

Aquagenic pruritus is characterized by an intense, burning discomfort of the skin which occurs after contact with water at any temperature or any degree of salinity. The symptoms normally begin during, or immediately after contact with water and last for about half an hour.[11] The buttocks and thighs are most commonly affected, and there is never any observable change in the appearance of the skin. Most cases are idiopathic, although some have been attributed to polycythaemia.[11]

Our patient has two rare manifestations of the HES, namely alcohol intolerance and aquagenic pruritus.[12] His response to photochemotherapy is of interest, since, although this was of little therapeutic value, it reduced the patient's circulating eosinophilia. There are two other case reports of successful use of PUVA in the HES,[8,13] and in both these cases the disease was limited to the skin. The mechanism by which PUVA reduced the circulating eosinophil count is unknown but possibly the stimulus to the eosinophilia arose in the skin, and the PUVA acted by reducing this stimulus. Alternatively, PUVA may have had a direct effect on eosinophils as they circulated through the skin. Studies are in progress to investigate this further.

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Mr Peter Deal kindly performed the immunocytochemistry on the skin specimen.

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Naevus corniculatus: a new acantholytic disorder

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SUMMARY

We describe a 33-year-old man with an unusual epidermal naevus that followed the lines of Blaschko. There were filiform hyperkeratoses, large cutaneous horns and lesions that resembled giant comedones and linear hyperkeratotic plaques. All of these lesions showed acantholysis without dyskeratosis. As the disorder is characterized by multiple small or large horn-like processes, we propose the term 'naevus corniculatus'.

Various types of epidermal naevi have been described, such as the common verrucous type, the epidermolytic type,[1] and the inflammatory linear verrucous epidermal naevus (ILVEN).[2] These are not simply variants of each other, but are regarded as being distinct entities and all of them have a linear arrangement that follows the lines of Blaschko.[3]

We report an epidermal naevus that differs from the types known so far, and for which we propose the term 'naevus corniculatus'.

CASE REPORT

A 33-year-old patient had a hyperkeratotic skin disorder from birth. The family history was non-contributory and the patient had a daughter who was normal. Physical examination revealed an epidermal naevus that was arranged in streaks and involved the entire body with the exception of the head (Fig. 1). The distribution of the skin lesions followed the lines of Blaschko. Most of the individual lesions consisted of horn-like processes (Fig. 2) and some areas were covered with filiform hyperkeratoses (Fig. 3). Other areas showed large cutaneous horns, measuring up to 2 cm. The patient was able to remove these horns without any pain, to leave a cavity lined by an erythematous keratotic wall. In other areas, the hyperkeratoses resembled giant comedones. Some of the lesions coalesced to form large linear verrucous plaques which were especially thick on the soles and were the cause of pain whilst walking. In 1970 some of the large hyperkeratotic lesions of the right sole had been removed surgically, with subsequent grafting of skin obtained from the right thigh.

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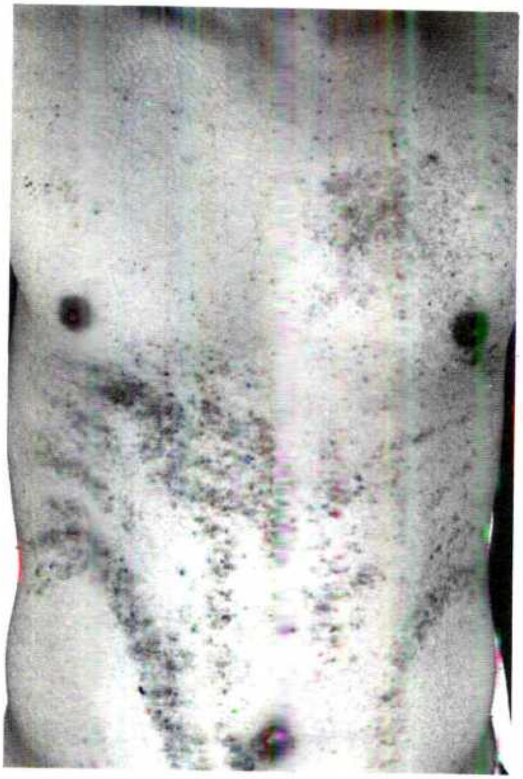

FIGURE 1. Naevus corniculatus, arranged in a systematized pattern following the lines of Blaschko.

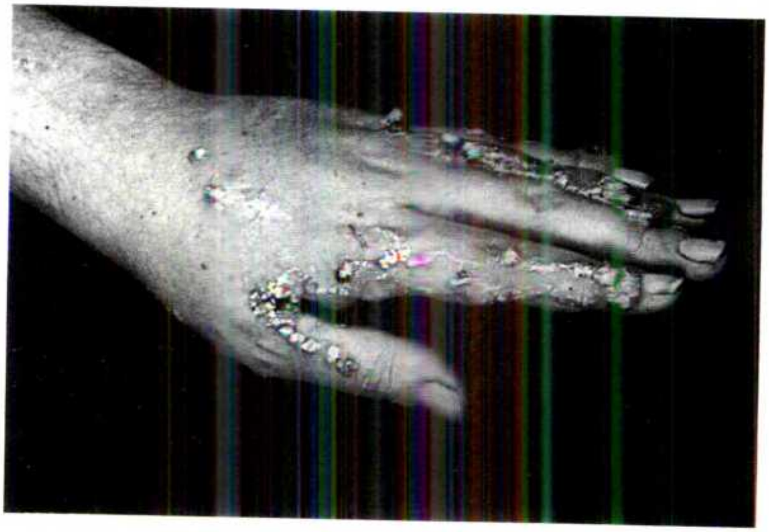

FIGURE 2. Linear arrangement of horn-like processes on the left hand.

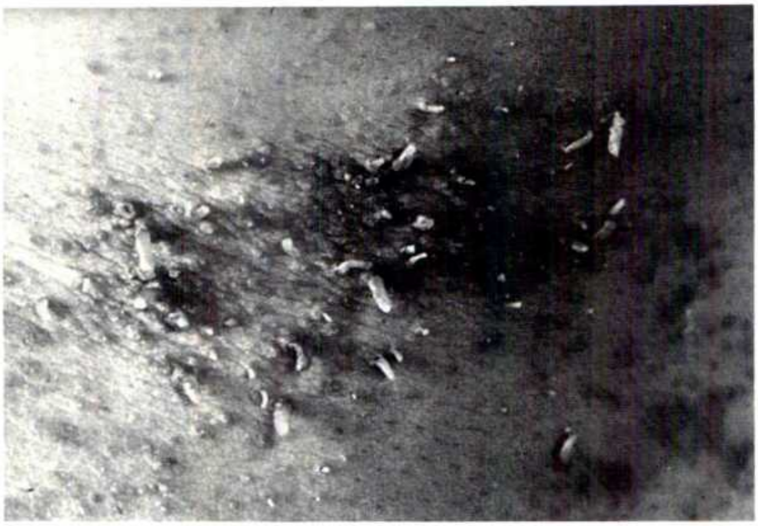

FIGURE 3. Filiform hyperkeratoses on the trunk.

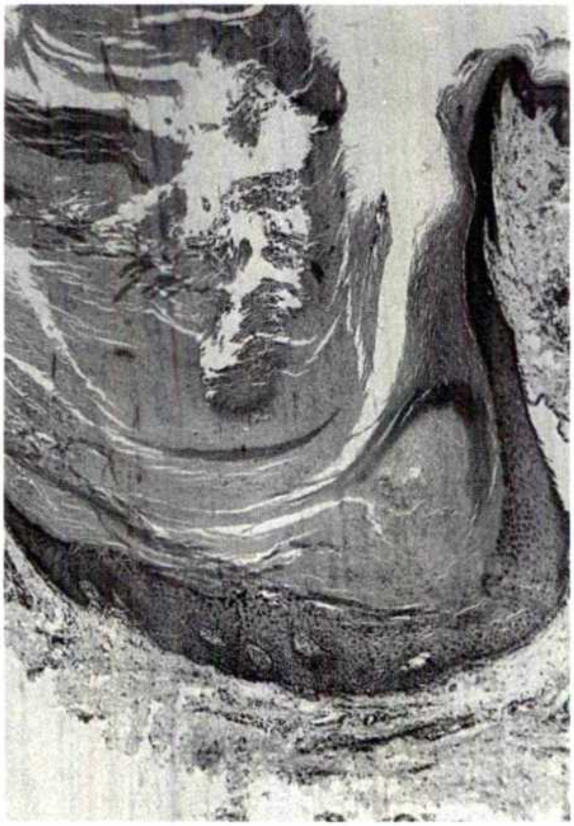

FIGURE 4. Cutaneous horn showing cleavage within the underlying epidermis (original × 30).

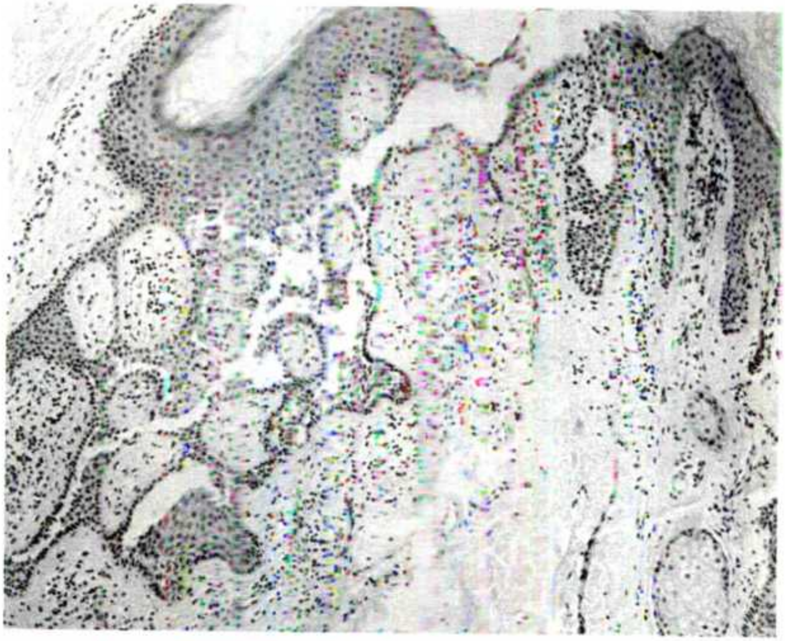

FIGURE 5. Intraepidermal cleavage with partial adhesion of the acantholytic prickle cells, giving the impression of a dilapidated brick wall (original × 80).

Light and electron microscopical findings

During the past 20 years, eight biopsies were taken of lesions from various sites. Light microscopy of these biopsies showed a thickened epidermis with marked hyperorthokeratosis (Fig. 4). The most prominent feature, however, was acantholysis with clefting both suprabasally and in the upper epidermis, resulting in the appearance of a 'dilapidated brick wall' (Fig. 5). Dyskeratotic cells were only rarely noted.

On electron microscopy the keratinocytes showed thickened tonofilament bundles as well as elongated and branching microvilli (Fig. 6 a–c). The wavy tonofilament aggregates surrounded the nuclei in a whorling configuration. The microvillous projections only rarely contained tonofilaments and desmosomal attachments. These changes were present in the suprabasal layer and were more prominent in the upper epidermis. Widening of the intercellular spaces, resulting in blister formation, was found throughout the entire epidermis. There was no dyskeratotic degeneration of the acantholytic cells. The granular layer showed a regular deposition of keratohyalin granules on the thickened tonofilaments. The horny layer was markedly thickened but did not show pathological inclusions.

DISCUSSION

This uncommon type of epidermal naevus was characterised by acantholysis and we were not able to find a similar case in the available literature. Clearly this naevus is neither Hailey–Hailey disease,[4] Darier's disease[5] nor familial dyskeratotic comedones.[6] The systematized linear

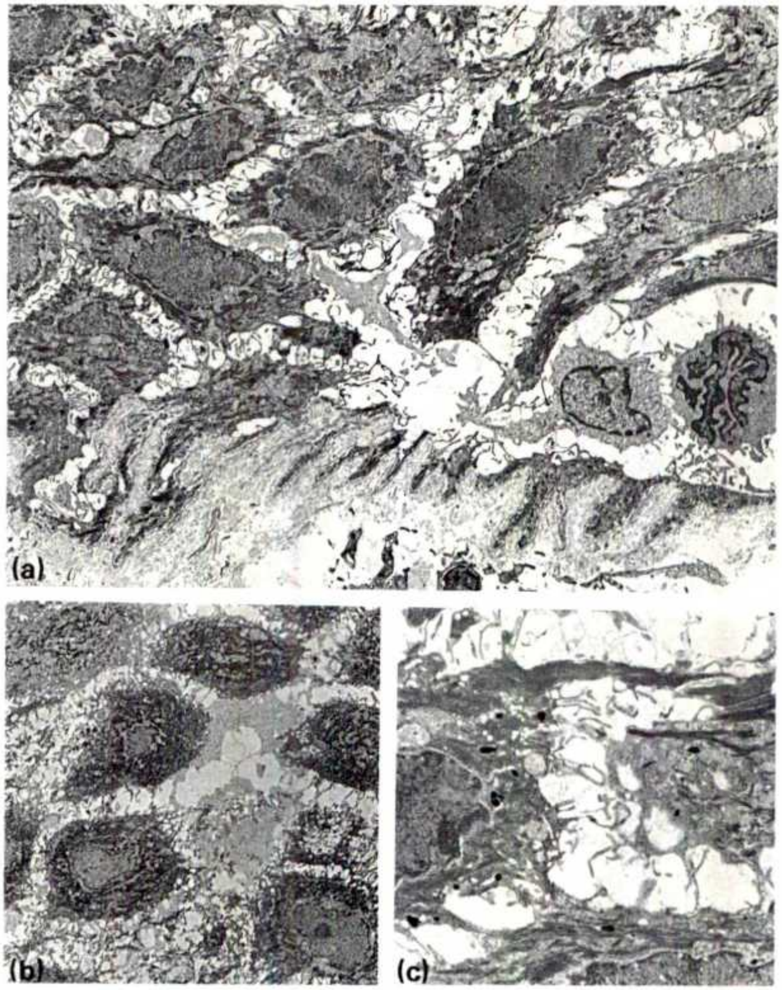

FIGURE 6.(a) Low-power electron microphotograph of the basal and suprabasal epidermis showing initial acantholysis with widened intercellular spaces (× 2800). (b) Acantholytic cells within the prickle cell layer showing characteristic disorganisation of tonofilament bundles (× 1400). (c) High power electron microphotograph of the atypical microvillous projections of keratinocytes. There are numerous elongated microvilli without tonofilament–desmosome complexes (× 6500).

distribution is similar to that observed in acantholytic dyskeratotic epidermal naevus.[7,8] In this condition, however, the individual lesions resemble those of Darier's disease[9,10] and there are neither filiform horns nor comedo-like lesions as observed in our patient. The light and electron microscopical findings of our case resembled those seen in Hailey–Hailey disease[11] rather than Darier's disease.[5] Our case is also fundamentally different from the relapsing linear acantholytic dermatosis as described by Vakilzadeh and Kolde,[12] the individual lesions being persistent and inflammation only rarely occurring.

In conclusion, this naevus seems to be a new skin disorder. Its clinical, histopathological and ultrastructural features separate it from any other genetic acantholytic skin disorders and, in particular, from other linear conditions such as relapsing linear acantholytic dermatosis and acantholytic dyskeratotic epidermal naevus.

As horn-like processes are the clinical hallmark of the disorder, we propose the term 'naevus corniculatus'. The word corniculatus means 'covered with small horn-like processes or horns'.[13] The fact that the cutaneous horns can reach such a considerable size despite acantholysis at their base, may be explained by the almost complete absence of inflammation. This contrasts with that seen in other acantholytic skin disorders though the phenomenon of acantholysis may explain why many of these horns can easily be removed without pain or bleeding.

It is likely that this naevus originated from a somatic mutation occurring at an early stage of embryogenesis, or from a gametic half chromatid mutation.[14] Whether the distribution is bilateral or unilateral, and whether the involvement is widespread or localized, will depend on the time during embryonic development when the somatic mutation occurred.

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British Journal of Dermatology (1990) **122**, 113–123.

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Topical cyclosporin A in psoriasis

SIR, There has been considerable interest recently in the efficacy of cyclosporin A (CsA), a potent immunosuppressive drug, in some inflammatory diseases. Several studies[1,2] have reported the beneficial effect of oral CsA on patients with psoriasis, including studies on short-term[3,4] and low-dose CsA therapy.[5–7] The effect of topical CsA in psoriasis has been investigated because of the dose-related side-effects of systemic CsA, particularly its nephrotoxicity. Previous studies on topical CsA in psoriasis failed to observe any clinical response,[8–10] but conversely, Powles *et al.*[11] have shown that intralesional injections of CsA may generate a local but not a systemic antipsoriatic effect.

We report the results of a bilateral comparison of topically applied CsA with placebo, using higher concentrations of CsA than in previous studies and applied under occlusion. This study on six patients with chronic plaque psoriasis had local ethical committee approval. The oral solution of CsA (Sandimmune®, Sandoz Ltd, Basle, Switzerland) was made up of 5% w/w in Decoderm ointment base® (Merck, Darmstadt, F.R.G.) and applied under plastic film occlusion to plaques of psoriasis for 6 h/day over 2–3 weeks. The placebo ointment containing only the solvent of CsA in the same vehicle was applied under identical conditions to corresponding lesions on the opposite side of the body. The lesions were scored for erythema, plaque thickness, and scaling on a 0–3 point scale at weekly intervals. Since the most sensitive indicators of the effect of systemic CsA on psoriatic lesions are the reduction of the cell infiltrate and the decrease of epidermal mitoses 1 week after onset of therapy,[1] we monitored the possibe topical effect of CsA on psoriasis by immunostaining of the cell infiltrate (Leu 1, Leu 2, Leu 3, CD22, HLA-DR, OKT6) and by autoradiography using the *in vitro* double labelling technique with [^{3}H]- and [^{14}C]-thymidine. The CsA level in pooled skin from treated lesions was measured using the standarized polyclonal radioimmunoassay-kit (Sandoz Ltd) in skin extracts. Before measurement the horny and upper layers of the epidermis were removed by a scalpel in order to measure only CsA absorbed in the lower epidermis and dermis.

One week after the onset of therapy the CsA concentration in CsA treated lesions was 3880 ng/g tissue wet weight. This equals the concentration in psoriatic lesions after 7 days of systemic therapy, which has been found to be 1·1 ± 0·3 ng/μg DNA.[12] All CsA treated lesions showed a marked decrease in the number of infiltrating neutrophils in the epidermis and adjacent papillary dermis. The mean ± SD of the pretreatment number of neutrophils per high power field was 8·2 ± 8·0 for the epidermis and 14·2 ± 10·2 for the papillary dermis, respectively, compared with 4·7 ± 4·4 and 5·7 ± 5·8 in CsA-treated lesions and 8·0 ± 7·6 and 12·5 ± 13·6 in placebo-treated lesions ($n = 6$ patients; $P < 0{\cdot}05$ using the 2-way Wilcoxon matched-pairs signed-rank test). The mononuclear cells of the epidermis, in particular the helper/suppressor T-cell ratio, were not affected. In spite of these results none of the six patients showed any specific clinical effect with CsA-treated lesions compared with control lesions. Side-effects were not observed. We also failed to demonstrate any effect of topical CsA on epidermal growth kinetics, at least not on the labelling index and the S-phase duration.

Our results show that topical CsA was absorbed in adequate quantities into the deeper epidermis and upper dermis and induced a biological response as shown by the decrease of neutrophils. However, the pathogenetic events responsible for inflammation and epidermal hyperplasia in psoriasis were not altered, and mononuclear cell infiltrates and epidermal kinetics remained unaffected. Apparently, the selective inhibitory effect on the neutrophils was not potent enough to clear the lesions. It may be that the absorption of topically applied CsA into the skin did not reach its biological target (vessels ?) in adequate concentrations, or that intraepidermal metabolism of CsA differs considerably from that in the liver. CsA is extensively metabolized in the liver by P450-dependent enzymes and it has been suggested that the metabolites 17 and 1 contribute to immunosuppression.[13] Perhaps topical therapy with these metabolites should be tried.

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Premenstrual exacerbation of atopic dermatitis: autoimmune progesterone dermatitis?

SIR, Dr Kemmett[1] observed premenstrual exacerbation of atopic dermatitis in a significant number of his patients but was unaware of previous reports of this phenomenon. Perimenstrual exacerbation of atopic dermatitis may be confused with autoimmune progesterone dermatitis.[2] This is a relatively rare disorder with only 25 cases having been described. Though it is usual in this condition for the rash to disappear completely during the follicular phase of the menstrual cycle, persistent disease with perimenstrual flare has been reported.[3]

We have treated two such patients both of whom, like other cases in the literature, demonstrated sensitivity to intradermally administered aqueous progesterone. It would be important to exclude this diagnosis in the group of women with premenstrual flare since ovulation suppression[4] provides an effective means of treating this distressing problem.

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Sneddon's syndrome and primary anti-phospholipid syndrome (PAPS)

SIR, The recent paper on Sneddon's syndrome by Grattan *et al.*[1] reports a patient with a long history of livedo reticularis complicated by a syndrome of painful leg ulcers, purpura and scarring. These are the typical features of livedo reticularis and atrophie blanche. She also developed incipient gangrene of a toe and transient ischaemic attacks. All the immunological tests in this patient with the exception of antibodies to cardiolipin and cryoglobulins were negative.

We have recently attempted to define particular subsets of patients demonstrating antiphospholipid antibodies,[2] who additionally manifest one or other features of the antiphospholipid syndrome, namely venous/arterial thromboses that may be accompanied by thrombocytopenia and less frequently by a positive Coomb's test or with frank haemolytic anaemia. Recurrent foetal loss from thrombotic occlusion of placental vessels occurs in many, and a small placenta may be indicative of some other aetiopathogenesis. Patients with a 'primary' antiphospholipid syndrome (PAPS), into which subset this patient appears to fall, are characterized by none of the major clinical manifestations of systemic lupus erythematosus such as skin rashes and photosensitivity, polyserositis, renal or non-cerebrovascular involvement. They do not have any of the acute features of SLE. They may frequently complain of arthralgias, which do not progress to arthritis, or migraines. The accent of their illness is on blood vessels, with all the attendant complications. Thus, deep vein thromboses, pulmonary thromboembolism, a variety of arterial occlusions, multi-infarct dementia as well as livedo reticularis may occur, just as in the antiphospholipid syndrome associated with full-blown SLE.

In our large multicentre study,[3] we sometimes found a family history of connective tissue disorders and that these patients might demonstrate a positive rheumatoid factor, cryoglobulins as well as have a false-positive test for syphilis (VDRL). The features of this syndrome have also been documented by others.[4,5] The second subset includes patients with 'lupus-like' disease, really a variant of lupus itself, who do not demonstrate four or more criteria for the diagnosis of SLE, as advised by the 1982 Committee,[6] but have lupus rashes of one kind or another and other clinical features of SLE.

It now seems clear that those patients previously referred to as Sneddon's syndrome[7] should be termed PAPS, if antiphospholipid antibodies are present. The term Sneddon's syndrome should be reserved for the majority of these patients who do not have antiphospholipid antibodies.[8,9]

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Hand erythema due to infusion of sympathomimetics

SIR, We wish to report a case of erythema of the hands due to an infusion of a sympathomimetic drug. A 29-year-old pregnant female, was in premature labour and was treated with an infusion of Albuterol 25 mg per day. Twenty-four hours after the onset of treatment with this drug, erythema of both palms was noted, which was patchy and only seen in places where glomus bodies are known to be abundant (Fig. 1).[1] The erythema regressed gradually after stopping the drug. The case suggests that β 2-adrenoreceptors are present in glomus bodies.

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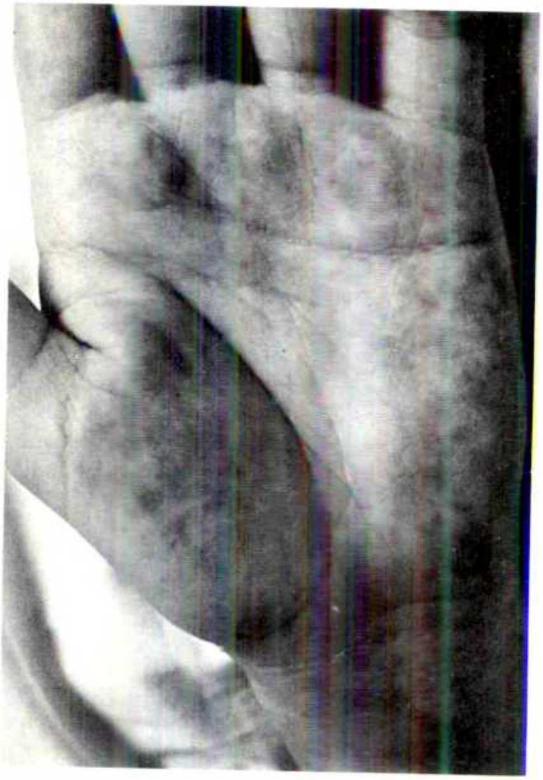

FIGURE 1. Volar hand erythema; the erythema is patchy and is located at places where glomus bodies are known to be found.

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Clinical and subclinical HSV infection resulting from exposure to asymptomatic patients

SIR, We report a case that exemplifies clinical and subclinical reinfection with herpes simplex virus (HSV) following exposure to a lesion-free partner, and the association of reinfection with reactivation of latent HSV.

A 28-year-old sexually active Caucasian woman was diagnosed as having a HSV infection in January 1987 and was treated with a 10-day course of oral acyclovir, 2g daily. The diagnosis of primary HSV type 2 (HSV-2) was confirmed serologically and sera obtained at the first visit and 3 weeks later were assayed for HSV-specific antibody using the highly sensitive antibody-typing multiplicity-analysis neutralization assay.[1] Antibody specific for HSV-2 was detected only in the convalescent serum. Both sera were negative for HSV type 1 (HSV-1) antibody.

When she was seen 2 months later the patient was free of clinical symptoms but still positive for antibody only to HSV-2. Her fiancé, who had no history of oral or genital HSV infections or other STD was seropositive only for HSV-1.

Three months later the patient experienced an anal tear following a water-skiing accident, without extension or trauma to the vagina. Before the rectal abrasions had completely healed, the couple engaged in oral and genital intercourse without the use of prophylactics. Five days later, the patient noticed a severe vulvo–vaginal rash, reminiscent of her first HSV episode, and returned to the clinic. This second episode involved both the pubic and perineal areas with ruptured vesicles particularly near the clitoris. The introitus and vagina were very inflamed. Fluid from 15 vesicles was cultured for virus and seven isolates obtained and typed with monoclonal antibodies specific for HSV-1 and HSV-2 (Syva Microtrak, Palo Alto, CA, U.S.A.). Two were HSV-1, one HSV-2 and four were a mixture of HSV-1 and HSV-2 (Table 1). Twelve plaque-purified isolates obtained from these latter four samples stained as HSV-1 (eight isolates) or HSV-2 (four isolates), consistent with the interpretation that clinical symptoms were due to a mixed infection with HSV-1 and HSV-2. Serum obtained at this time was positive only for presence of HSV-2 antibody, whereas serum obtained 4 weeks later had antibody to both HSV-2 and HSV-1. The partner's serum was positive only for HSV-1 antibody.

The couple was seen again 7 months later and during this period there were no genital or oral lesions and they claimed to have had no other sexual partners. At this visit, the patient's partner demonstrated seroconversion to HSV-2 antibody indicative of subclinical infection with HSV-2.

There has been a significant increase in the incidence and prevalence of HSV infections during the last two decades.[2] Primary infection often occurs following exposure to asymptomatic individuals. Reinfection is not well recognized, and its risk and clinical outcome as well as its potential effect on a previously established latent HSV infection remain unclear. Experimental HSV reinfection in the presence of apparently normal virus-specific immunity has been described[3] and HSV-1 or HSV-2 were isolated from different recurrent episodes at the same site.[4] In rare cases both HSV-1 and HSV-2 were simultaneously isolated from genital lesions[5] but the source and temporal relationship of infection with the two HSV types were not clearly established.

This couple demonstrated both clinical and subclinical infection resulting from exposure to asymptomatic patients, and trauma-related reinfection apparently accompanied by reactivation of the initial latent virus and resulting in severe clinical symptoms. Our findings suggest that immunity characterized by the presence of HSV-specific neutralizing antibody, does not protect from subsequent reinfection with the heterologous HSV type. Additionally, we find that reinfection can be asymptomatic or it can be associated with severe symptoms, not unlike those seen during the primary HSV-2 infection.

Conclusive proof that the HSV-2 isolated from the patient's second episode is the same as that in the primary infection, and that the HSV-1 is the same as that of her sexual partner would require restriction enzyme fingerprinting of viral DNA extracted from the different isolates. Nevertheless, it seems reasonable to assume that the HSV-2 in the second disease episode was of endogenous origin (reactivated virus), since the partner was HSV-2 seronegative and the couple were monogamous. Based on the same criteria, we assume that the HSV-1 infection was acquired from her partner. The history of oral sex before the trauma-induced skin abrasions had healed, is consistent with this interpretation. Presumably the increased risk of reinfection, the severity of the lesions and the reactivation of the latent HSV-2 are related to the local trauma, since trauma was shown to cause a significant reduction in the titre of virus required to cause severe and/or lethal HSV infections.[6] Additionally, local trauma has been associated with the induction of suppressor T (Ts) lymphocytes and suppressor factors that mediate decreased resistance to cutaneous infection, and Ts-mediated inhibition of HSV-specific CMI responses was shown to accompany HSV reactivation and recurrent disease.[7] However, our findings cannot differentiate between the contribution of the local trauma and that of HSV-1 reinfection to the apparent reactivation of the latent HSV-2. Consistent with our previous suggestion,[8] this couple provides compelling evidence that infection/reinfection is not a rare event and often results from exposure to asymptomatic virus shedders.

TABLE 1. Virus isolated from multiple vesicles at the time of secondary infection

Vesicle no.	Virus isolated	Virus type*	Plaque purified virus
1	+	HSV-1	ND
2	+	HSV-2	ND
3	–	–	–
4	–	–	–
5	+	HSV-1/2	HSV-2 (1);†HSV-1 (2)
6	–	–	–
7	–	–	–
8	+	HSV-1/2	HSV-2 (1); HSV-1 (2)
9	–	–	–
10	+	HSV-1/2	HSV-2 (1); HSV-1 (3)
11	+	HSV-1/2	HSV-2 (1); HSV-1 (1)
12	–	–	–
13	–	–	–
14	–	–	–
15	–	HSV-1	ND

* Typing was done by immunofluorescent staining of infected MRC-5 cells with monoclonal antibodies specific for HSV-1 or HSV-2 (Syva Microtrak, Palo Alto, CA U.S.A.).

† Parentheses represent the number of plaque purified HSV isolates.

ND–not done.

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Spontaneous regression of infantile digital fibromatosis

SIR, I read with interest the case report by Ishii and colleagues of infantile digital fibromatosis in which spontaneous regression was beautifully illustrated.[1] I agree with the authors that spontaneous regression with time is the rule, and have previously reported further evidence of this.[2] In addition to the case of Ishii and colleagues and the five cited by them, among our 30 cases we described one with spontaneous regression and found 15 in the literature. The importance of these observations lies in the fact that although recurrences may be alarmingly rapid, large and aggressive, extending down to periosteum, treatment should be conservative. As metastasis has not been recorded, the amputations sometimes performed in the past (38 of 115 cases to 1985[2]) can no longer be justified.

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Necrotizing vasculitis and severe atherosclerosis: resolution of the vasculitis following surgery

SIR, We were interested in the recent report by Alegre and Winklemann[1] in which five patients were described with severe atherosclerosis and a necrotizing vasculitis and which suggested that the relationship between the two disorders changes and exacerbates the clinical and pathological expression of each disease. Recently we observed such an association where the vasculitis revealed the existence of severe atherosclerosis in both lower limbs.

A 65-year-old woman was seen in March 1988 with multiple, small-ulcerated lesions and erythematous purpuric papules on the lower legs (Fig. 1). The lesions rapidly developed into areas of necrosis and the patient suffered a considerable amount of pain. A biopsy showed a small vessel leucocytoclastic vasculitis with fibrinoid necrosis and fibrohyalinosis and an arteritis obliterans of the deeper vessels. There was no evidence of cholesterol microembolization and investigations revealed no signs of infection or of collagen diseases and neoplasia. There was no iatrogenic cause for the vasculitis. Arteriography of the lower limbs showed thromboses of the left superficial femoral and popliteal arteries and in the right leg diffuse and severe atheroma of the iliac and femoral arteries and emboli at the level of the superficial femoral and tibial arteries. A left femoral to anterior tibial artery bypass was performed, using the *in situ* saphenous vein as a graft. Operative arteriography confirmed the patency of the graft and a satisfactory distal anastamosis. Post-operatively the patient was started on anticoagulants with initially intravenous heparin and later with a coumarine derivative. Within 2 weeks following the operation there was a dramatic improvement of the vasculitis (Fig. 2) and marked relief of her pain. Six months afterwards the patient was still on anticoagulant therapy, but there was no recurrence of her vasculitis.

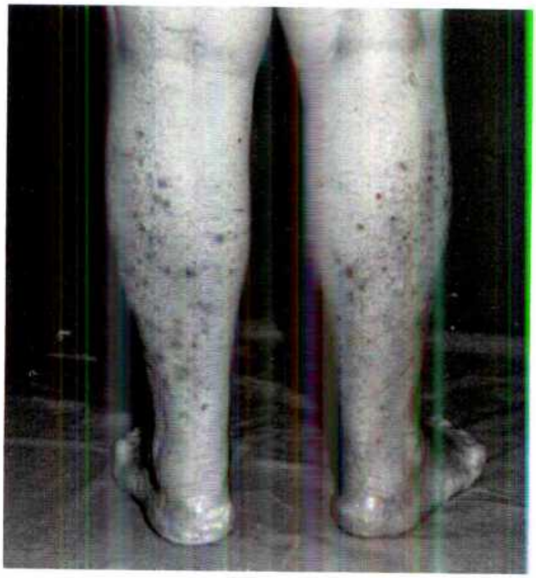

FIGURE 1. Multiple small ulcerated lesions on the lower legs associated with purpuric papules.

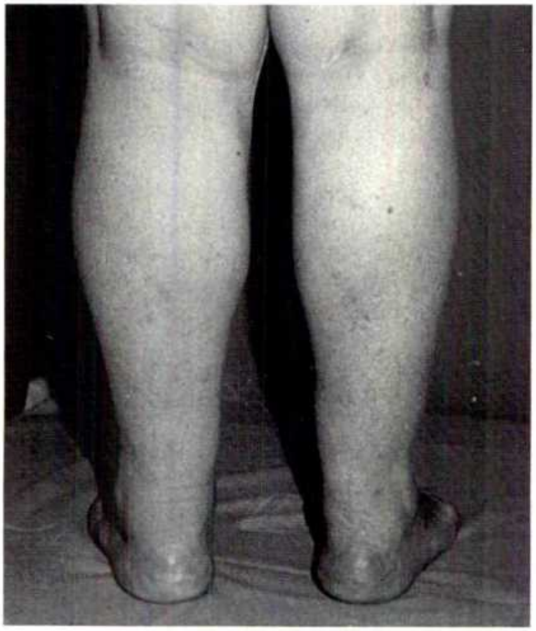

FIGURE 2. Resolution of the vasculitis, 20 days after surgery.

This case emphasizes that a necrotizing vasculitis can reveal severe and complicated atherosclerosis and where possible, rapid surgical treatment can arrest the development of the vasculitis.

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Cyclosporin A for treatment of severe lichen planus

SIR, There is evidence that lichen planus (LP) is associated with an alteration of a cell-mediated immune response[1] and that the majority of the infiltrating mononuclear cells in the dermis are $CD4^+$ and $CD1^+$.[2] Lichen planus-like eruptions are commonly seen in bone marrow transplant patients with graft-versus-host reactions.[3]

We have studied eight patients (seven male, one female) with severe, extensive LP with recent onset and who have been treated over a short period of time with oral cyclosporin (CyA). The patients had no previous history of neoplastic disorders and there was no clinical evidence of any active infections. They had not received any other systemic treatment for at least 2 months, and had not used any topical treatments apart from emollients in the 2 weeks prior to therapy with CyA. Before treatment the blood pressure, blood creatinine, serum electrolytes and liver function tests were determined, as well as a full blood count, and these were all normal. The CyA was given orally once a day, and the dose and duration of treatment with this drug for each patient are shown in Table 1. The plasma cyclosporin concentration was determined by the Sandoz radioimmunoassay as a plasma trough level 12 h after the last dose, and this was measured each week. At clearance of the lesions or after 5 weeks, therapy was discontinued and all patients were examined monthly for up to 6 months after stopping the drug to determine whether their LP had recurred.

Seven of the 8 LP patients were clear of their lesions on a dose of CyA of 3 mg/kg per day. The time for resolution of their lesions varied from 10 days to 3 weeks, but a significant improvement was seen within 1–2 weeks. In only one patient with a dose producing a CyA trough level of less than 100 ng/ml was it ineffective, but in this patient an increase in the dose from 3 to 5 mg/kg per day produced an improvement. However, we could not evaluate this because of some loss of renal function and hypertension. This necessitated stopping treatment. Other reversible adverse effects in our patients were hypertriglyceridemia and nausea. Several patients had transitory deterioration of renal function with some elevation of their creatinine and blood urea levels and their creatinine clearances.

Cyclosporin has been used for a number of skin disorders, including not only psoriasis[4] but ichthyosis, pemphigus, Behçet's disease, mycosis fungoides, systemic lupus erythematosus and alopecia areata.[5,6] However, we have found only one report of its use in LP, in the treatment of oral erosive lesions.[7] In our study, we have found that CyA is an effective agent for the treatment of severe LP over a short period of time. The remission induced by CyA can then be maintained after stopping the drug with other treatments such as topical steriods.

TABLE 1. Clinical profiles in patients with LP treated with CyA

Patients	Age	Sex	Area of involvement %	CyA dosage mg/kg per day	Time for clearing	Follow-up to 6 months	Side-effects
1	27	M	30	3	2 weeks	Remission with topical steroid	None
2	50	M	40	3	2 weeks	Remission with tar	Increase in triglyceride levels
3	50	M	30	3	3 weeks	Remission with UVA + B	None
4	50	M	30	3	10 days	Remission	None
5	30	M	25	3	—	—	Creatininaemia, hypertriglyceridaemia
6	62	F	20	3	3 weeks	Remission	Mild nausea
7	61	M	15	3	8 days	Remission	None
8	64	M	40	3	7 days	Remission with topical steroid	None

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British Journal of Dermatology (1990) **122**, 124.

Book Reviews

Oxford Handbook of Clinical Specialities. J.A.B.Collier and J.M.Longmore (1989). Oxford: Oxford University Press. Pp. 787 ISBN 0-19-261816-4. Price £10·95.

This is the second edition of this text which aims to assist junior medical students by being a portable reference, to gain more from their clinical experiences. General practice trainees are another group who, moving rapidly from one speciality to another, may find this book useful.

It is divided into 10 chapters comprising obstetrics, gynaecology, paediatrics, psychiatry, general practice, opthalmology, E.N.T., dermatology, orthopaedics and eponymous syndromes. Each topic usually has a page devoted to it and the comprehensive index together with indexing at the beginning of each chapter make for quick reference. A large number of blank pages are provided (approx 300) to allow the reader to make their own notes and attach the update boxes produced monthly in the B.M.J. in conjunction with Oxford University Press.

The dermatology chapter is short at only 29 pages and as the authors accept it needs to be used in conjunction with clinical experience or a good atlas of dermatology. Indeed it is so short as to be of little value except to a junior medical student prior to starting their dermatology attachment. Had there been more detail as in other chapters, with more emphasis on management and attention given to prescribing, it would provide a better reference facility. I was pleasantly surprised though to find a whole page devoted to graduated support hosiery. Included in this were useful tips on prescribing and as a reference page this must surely be the best in this chapter.

The bulk of the book is well presented, with diagrams where they will help, and crisp presentation of the information. Much detail has gone into most of the chapters and nearly all topics are well-covered. On the whole I think the book has achieved its aim. It does not aim to be a comprehensive reference as this would involve a vast increase in size thus loosing the ability to fit in a white coat pocket. I would personally consider it an additional book rather than a substitute for one of the standard references either on specialities or individual subjects. At £10·95 it is not expensive and for a junior doctor sitting in casualty or in the labour ward late at night it will provide a comforting bulge in their pocket.

W.Perkins

A Colour Atlas of AIDS in the Tropics. M.A.Ansary, S.K.Hira, C.Chintu and S.L.Nyaywa (1989). London: Wolfe Medical Publications Ltd. ISBN 0-7234-1567-6. Price. £35.00.

This is a beautifully illustrated hardback atlas in the Wolfe series. It contains a wealth of at times disturbing photographic material and is predominantly clinical. The range of problems which may affect the AIDS sufferer in tropical countries is quite remarkable. The book is thoroughly recommended as a probably unique and relatively inexpensive source of material in this expanding field.

R.M.MacKie

British Journal of Dermatology (1990) **122,** 125.

News and Notices

British Dermatological Surgery Group
Advanced Dermatological Surgery Workshop

The British Dermatological Surgery Group have organized a 2-day workshop in dermatological surgery 5–6 July, 1990, in Newcastle upon Tyne. This is for dermatologists interested in obtaining experience in the surgical aspect of the speciality and preference will be given to those who have previously attended one of the more basic skin surgery workshops. The topics that will be covered include, anatomy of the head and neck with cadaver specimens, skin grafting with pig's skin, Mohs surgery, repairs on the nose, mistakes and how to avoid them, liposuction, collagen implants, sclerotherapy, nail surgery and face peels. The lecturers will include Dr Larry Field, Professor of Dermatologic Surgery of the University of California who is the first holder of a Travelling Professorship of the International Society of Dermatological Surgery. The course fee is £180 which will include meals and accommodation provided in a University Hall of Residence. For further information please contact Mrs Ann Winstanley, Department of Dermatology, Royal Victoria Infirmary, Newcastle upon Tyne NE1 4LP, U.K.

British Society for Paediatric Dermatology
16 February 1990

A 1-day symposium on Atopic Eczema will be held at the Institute of Child Health in London on 16 February 1990. For further details contact Dr John Harper, Department of Dermatology, the Hospital for Sick Children, Great Ormond Street, London WC1N 3JH.

8th International Symposium on Bioengineering and the Skin
Stresa, Italy 13–16 June 1990

The 8th International Symposium on Bioengineering and the Skin is to be held at Stresa, Lake Maggiore, Italy on 13–16 June 1990. The local organisers are G.Rabbiosi, S.Sacchi, G.Borroni and E.Berardesca of the Department of Dermatology, University of Pavia, 27100 Pavia, Italy.

Dermatologists, pharmacologists, plastic surgeons, cosmetic chemists and other scientists will discuss the technologies and methods for the non-invasive evaluation of skin physiology, pathology, therapy and skin product assessment. The deadline for abstracts is 31 January 1990. Abstracts should be typed on A4 paper with a one inch margin on the left, and should include title, author's name and affiliation and should not exceed 300 words. Abstracts should be sent to Dr Jorgen Serup, Department of Dermatology, Rigshospitalet H 5132, 9 Blegdamsvej, 2100 Copenhagen, Denmark, or to Dr Randy Wickett, S.C.Johnson and Son, 1520 Howe Street, Racine WI 53403-5011, U.S.A.

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Human normal-resting epidermal Langerhans cells do express the type 3 complement receptor

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SUMMARY

The expression of CR3 by murine-resting epidermal Langerhans cells (LC) is well established, but CR3 expression by human normal-resting epidermal LC has not yet been demonstrated. In this study, highly sensitive immunostaining techniques, such as immunogold labelling in transmission- and scanning-electron microscopy, were used on freshly isolated, LC-enriched, normal human epidermal cells. Human normal resting epidermal LC were found to be $CR3^+$, since a low but significant number of gold granules labelled the plasma membrane of all the LC observed under transmission-electron microscopy, and all the epidermal cells showing LC morphology as observed by scanning-electron microscopy.

The type 3 complement receptor (CR3), which is the cell surface receptor for the complement cleavage product C3bi,[1] is a heterodimer composed of an unique α-subunit of 165 kDa non-covalently associated to a β-subunit of 95 kDa, the latter being shared by two other heterodimers belonging to the same glycoprotein family.[2]

It is now clear that murine-resting epidermal Langerhans cells (LC) express CR3.[3,4] In man however, the expression of CR3 by resting epidermal LC has not yet been demonstrated. $CD1a^+$ mononuclear cells, isolated from human bone marrow,[5] cord blood[6] or from the blood of burns patients,[7] and thought to represent LC precursors, are $CR3^+$. In histiocytosis X, which is known to represent a proliferative disorder of cells of LC lineage,[8,9] the cells (HXC) are $CR3^+$.[8–12] The majority of studies, especially those based upon immunohistology, suggest that human normal-resting epidermal LC are $CR3^-$.[8,10,11,13–15]

In this study we investigated CR3 expression by human normal-resting epidermal LC, by using highly sensitive immunostaining procedures such as pre-embedding immunogold techniques both on transmission-electron microscopy (IG-TEM)[16,17] and with scanning-electron microscopy (IG-SEM).[18]

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TABLE 1. Monoclonal antibodies (mAb) used to detect CR3

CR3 subunits	mAb	Source: OW (code number)*	Source: Commercial
α-chain	60·1	M45	
α-chain	D-12	M46	
α-chain	Vim-12	M49	
α-chain	anti-C3bi		Dakopatts
β-chain	60·3	M06	

*OW, Oxford Workshop on human leucocyte differentiation antigens (Oxford, 1986).

METHODS

Normal human skin was obtained from plastic surgery reduction operations. *In situ* studies were carried out by light (LM) and fluorescence (FM) microscopy and freshly isolated epidermal cells were investigated by immunoelectron microscopy (IEM). The monoclonal antibodies (mAbs) used, detecting the α- or β-subunit of CR3, are listed in Table 1.

In situ studies by LM and FM

Immunoperoxidase indirect labelling was performed on vertical cryostat skin sections, as previously described.[19] In the first layer mAbs were used, and in the second layer a peroxidase-conjugated goat anti-mouse IgG antiserum (Dakopatts, Glostrup, Denmark) was used.

Standard FM indirect immunostainings[20] were carried out on epidermal sheets obtained by mild trypsinization. In the first layer mAbs were used, and in the second layer a fluorescein-conjugated goat anti-mouse IgG antibody (Tago Inc., Burlingame, CA, U.S.A.) was used.

Fresh isolation of LC

Suspensions of freshly isolated epidermal cells and LC enrichment were made as previously described.[21] Briefly, skin slices were trypsinized using 0·25% trypsin in Hank's balanced salt solution (Gibco, Paisley, Scotland) for 30–45 min at 37°C, and the sheets were shaken and pipetted. The epidermal cell suspensions were centrifuged, resuspended in RPMI-1640 with 10% heat-inactivated fetal calf serum (Gibco), layered on a Ficoll-Hypaque gradient, and centrifuged. Such a LC enrichment technique achieved a good degree of purification with about 15% of the cells being LC. LC-enriched cells were collected from the interface, and resuspended in RPMI-1640 with 10% fetal calf serum.

IG-TEM studies on freshly isolated LC

LC-enriched, freshly isolated (as above) epidermal cells were prefixed with 0·1% glutaraldehyde, washed in PBS-0·1% bovine serum albumin (Sigma, St Louis, MO, U.S.A.), and, after a blocking pretreatment with 0·2% sodium azide, 1% bovine serum albumin and 10% decomplemented human AB serum, incubated with mAbs. A 15-nm sized gold-conjugated goat anti-mouse Ig antibody (Janssen Pharmaceutica, Beerse, Belgium) was used as a secondary antibody, as previously described.[17] Samples were prepared for transmission-electron microscopy studies by fixation in 1% glutaraldehyde, packaging in Bacto-Agar (Difco Laboratories, Detroit, MI, U.S.A.), post-fixation in potassium ferrocyanide-reduced osmium tetroxide, dehydration in acetone, embedding in Durcupan ACM (Fluka, Buchs, Switzerland), sectioning by a LKB III ultramicrotome, and examination with a Philips EM 300 electron microscope.

The density per cell section (midplane) of gold particles bound to the plasma membrane of LC labelled with the mAbs detecting the CR3 subunits was evaluated, assuming 50 LC per mAb.

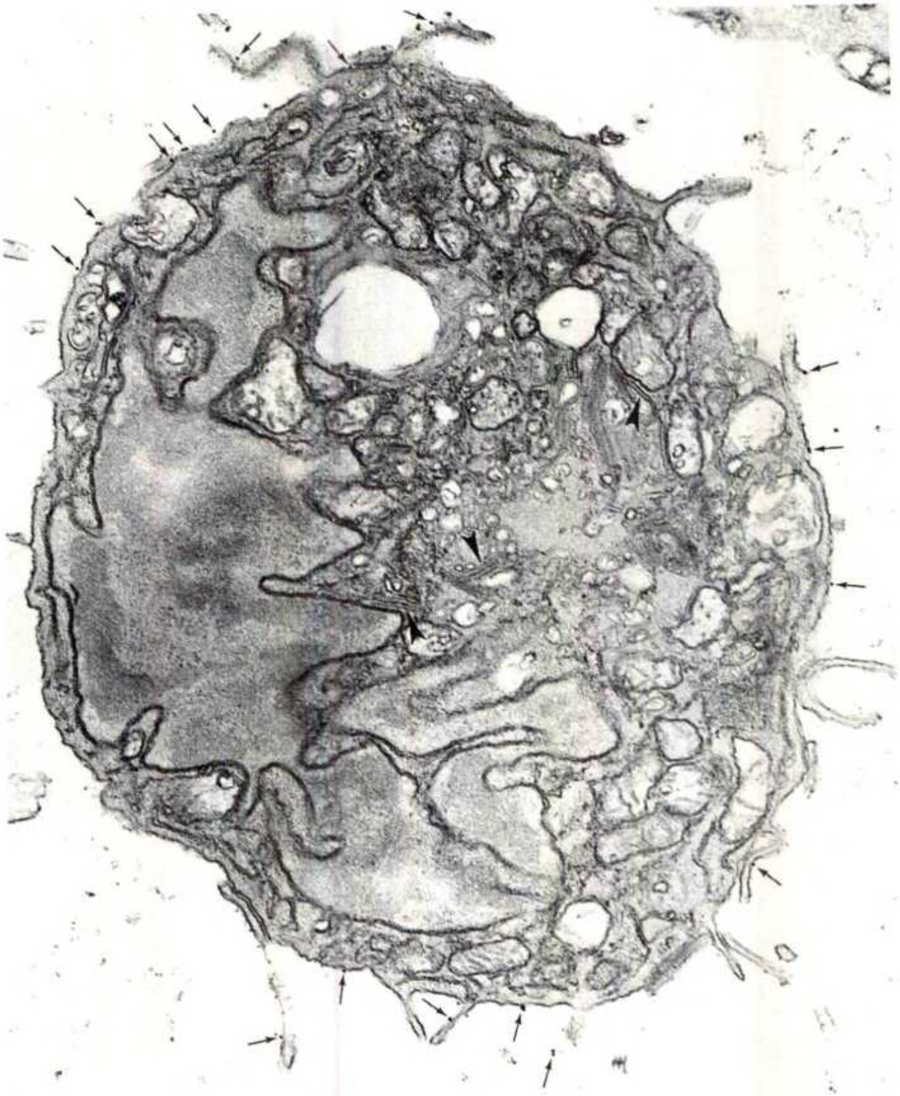

FIGURE 1. $CR3^+$ Langerhans cell. Immunolabelling with 60·1 mAb and 15 nm gold particles in IG-TEM. The arrows indicate the gold particles along the cell surface; the arrowheads indicate the Birbeck granules within the cytoplasm (× 16 800).

IG-SEM studies on freshly isolated LC

LC-enriched, freshly isolated epidermal cells were placed on 0·1% poly-1-lysine-pretreated glass coverslips, prefixed with 0·2% glutaraldehyde, subjected to a blocking pretreatment as above, incubated with mAbs in the first layer, and with a 40-nm sized gold-conjugated goat anti-mouse Ig antibody (Janssen) in the second layer.[18] After post-fixation in 2% glutaraldehyde, the samples were dehydrated, critical-point dried from CO_2, and coated with evaporated carbon (20 nm). The samples were then examined by a Philips 505 scanning-electron microscope, capable not only of secondary electron imaging (SEI) detection, but also equipped with a 4-scintillator

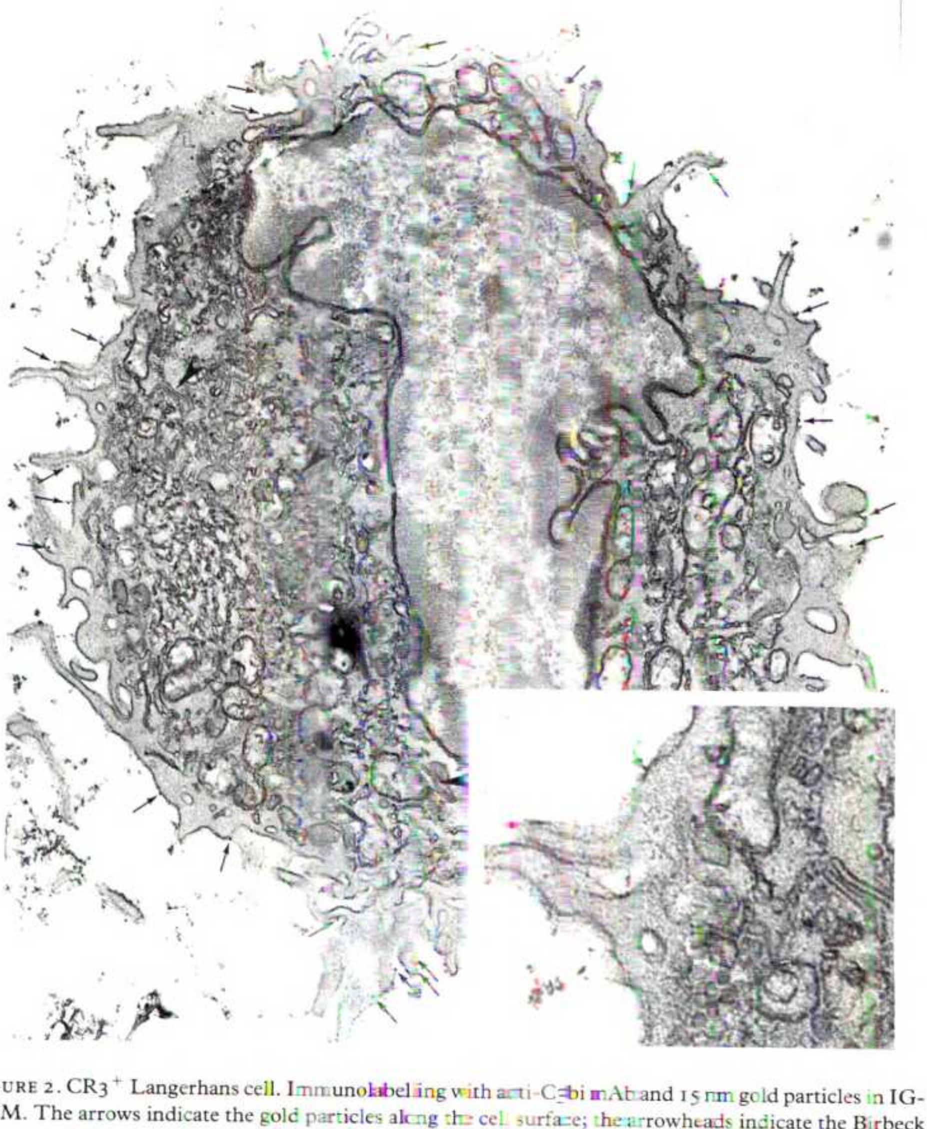

FIGURE 2. CR3$^+$ Langerhans cell. Immunolabelling with anti-C3bi mAb and 15 nm gold particles in IG-TEM. The arrows indicate the gold particles along the cell surface; the arrowheads indicate the Birbeck granules within the cytoplasm (× 15 400). Inset: high magnification, showing Birbeck granules in the cytoplasm and one gold particle on the surface (× 46 200).

type backscattered electron imaging (BEI) detector. Both secondary and backscattered electron signals were detected; reverse signal polarity was used for the BEI observations.[18]

Positive controls

The positive controls were performed using anti-CD1a mAbs instead of the anti-CR3 mAbs, both in LM/FM and in IG-TEM/IG-SEM. A blocking pre-incubation with anti-CD1a was performed both *in situ* (by LM and FM) and on freshly isolated, LC-enriched, epidermal cells (in IG-TEM and in IG-SEM), before incubating by anti-CR3 mAbs. To positively control the anti-CR3 mAbs, *in situ* LM and FM experiments were performed on normal human lymph node;[22] and the IG-TEM and IG-SEM methods were carried out on normal peripheral blood mononuclear cells, to detect the CR3 α-subunit positivity of monocytes, large granular lymphocytes and of a small subset of CD8 lymphocytes, and the β-subunit positivity of most

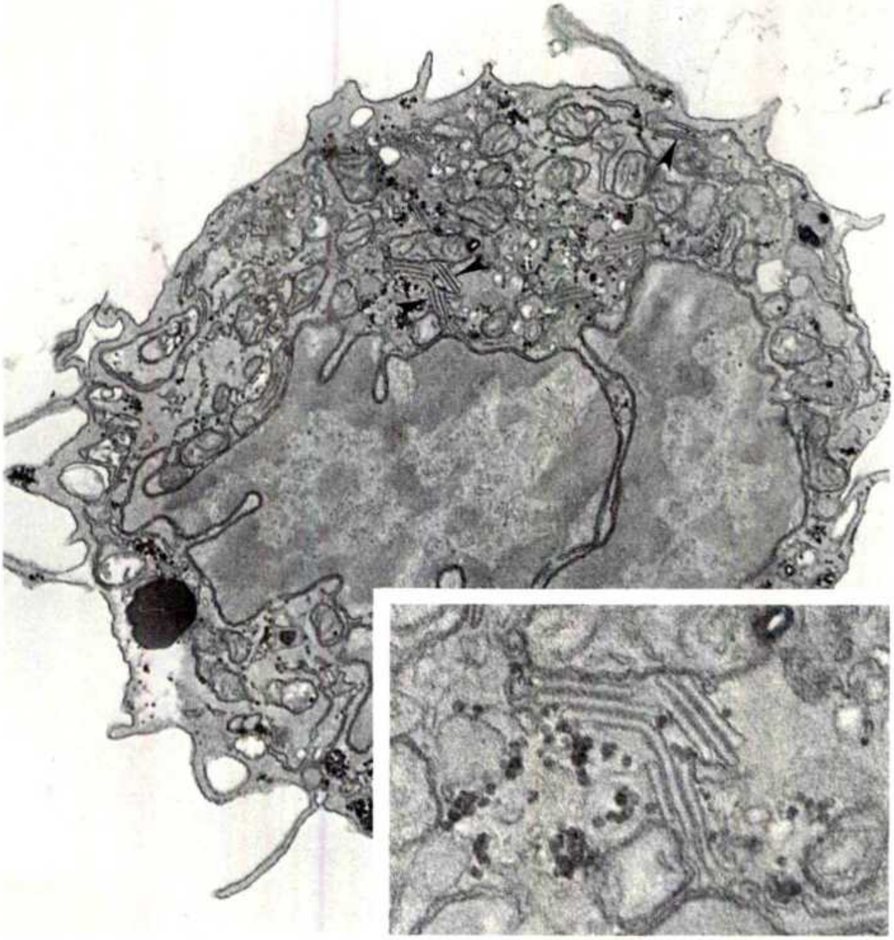

FIGURE 3. Negative staining (control for method specificity) of a Langerhans cell. Immunolabelling with mouse unreactive Ig of appropriate isotype instead of mAbs, in IG-TEM. No gold particles are visible along the cell surface; the arrowheads indicate the Birbeck granules within the cytoplasm (× 16 800). Inset: high magnification showing Birbeck granules in the cytoplasm (× 50 400).

peripheral blood mononuclear cells. Finally, an *in situ* IG-TEM technique previously described by us[23,24] was applied to normal skin sections to verify the CR3 positivity of LC *in situ*.

Negative controls

The specificity of the double-step procedures, performed in LM, FM, IG-TEM and IG-SEM, was assessed by: (i) inhibiting the labelling by pre-incubation, before the conjugated goat anti-mouse Ig antibody (second step), with an unconjugated goat anti-mouse Ig antibody; (ii) omitting the mAb (first step); (iii) substituting the mAbs (first step) by a mouse-unreactive Ig of appropriate isotype.

RESULTS

Immunolabellings by LM and FM

Both on vertical cryostat skin sections and on epidermal sheets, only a few dendritic epidermal cells were faintly visualized.

Immunolabelling of LC by IG-TEM

With transmission-electron microscopy (Figs 1, 2 and 3) labelling was detected with variable numbers of gold particles apposed to the plasma membrane (Figs 1 and 2). A cell was considered positive when at least three gold particles were attached to the cell surface per section.[25] All the mAbs, both the anti-CR3 α-subunit (four) mAbs and the anti-CR3 β-subunit (one) mAb, stained all the observed Birbeck granules-bearing LC, although the number of gold granules per LC section (midplane) varied (Table 2). Evaluation of the labelling showed that the LC were both CR3 α-subunit-positive (gold particles per LC section $7{\cdot}5 \pm 9{\cdot}5$; $n = 200$), and CR3 β-subunit-positive (gold particles per LC section $8{\cdot}0 \pm 4{\cdot}4$; $n = 50$). However, lower numbers of gold particles were observed on the surface of the CR3$^+$ LC, as compared to CD1a$^+$ control LC, as previously found.[16,17,21,24] The keratinocytes were CR3$^-$.

Immunolabelling of LC by IG-SEM

In the SEI mode, cells showing the morphological characteristic of keratinocytes were negative, whilst cells showing the morphology of LC were both CR3 α-subunit- (Fig. 4a) and CR3 β-

TABLE 2. Quantitative evaluation (mean ± standard deviation) in IG-TEM of colloidal gold granules linked to the surface of LC (midplane) stained with anti-CR3 mAbs (50 LC/mAb)

mAb	Number of gold granules
60·1	8·1 ± 4·3
D-12	5·3 ± 5·8
Vim-12	3·9 ± 2·9
Anti-C3bi	12·9 ± 15·4
60·3	8·0 ± 4·4

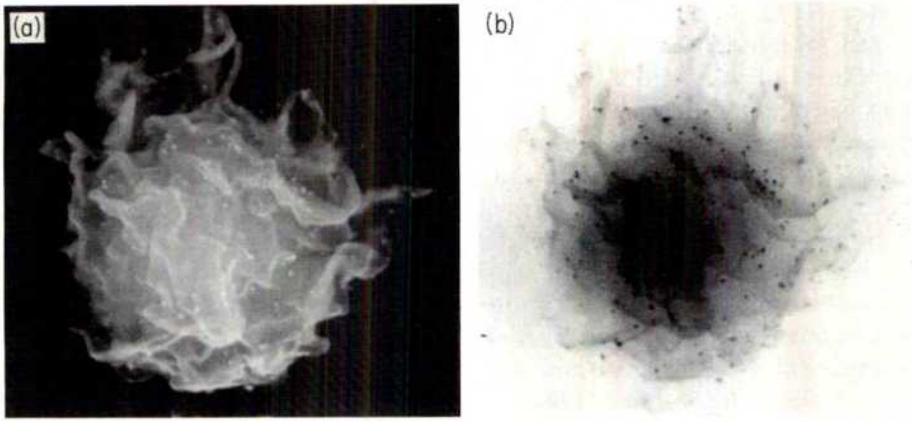

FIGURE 4. CR3 positive Langerhans cell. Immunolabelling with D-12 mAb and 40 nm gold particles in IG-SEM. (a) SEI mode. The morphological details of the Langerhans cell are clear. Gold particles (white dots) are faintly visible, on the cell surface (× 6300). (b) BEI mode; reversed signal polarity showing the same Langerhans cell as in (a). Although the structural details of the surface morphology cannot be seen, gold particles (black dots) are clearly visible (× 6300).

subunit-positive, with numerous gold particles evenly distributed as white dots on the cell surface (Fig. 4a). However, gold particles were more easily detectable in the BEI mode with reversed signal polarity as black dots (Fig. 4b), confirming their CR3 positivity.

Controls

All the immunostainings showed CD1a-positivity of dendritic epidermal cells (LM and FM) and of Birbeck granules bearing LC (IG-TEM and IG-SEM). The pre-incubation with anti-CD1a mAb did not block the reactivity of anti-CR3 mAbs with LC. Positive controls using sections of normal human lymph node (LM and FM) or peripheral blood mononuclear cells (IG-TEM and IG-SEM) were positive, since anti-CR3 β-subunit mAb stained most mononuclear cells, whereas anti-CR3 α-subunit mAbs stained only monocytes, large granular lymphocytes and a small subset of lymphocytes.[26]

The *in situ* pre-embedding IG-TEM control technique confirmed the CR3 positivity of LC on vertical sections of normal human skin, as recently reported by us.[27] All the controls for the specificity of the two layers procedures, both in LM/FM and in IG-TEM/IG-SEM, gave negative results (Fig. 3).

DISCUSSION

Our results show that freshly isolated human epidermal LC express not only the CR3 β-subunit, which is not exclusive for CR3,[2] but also the highly CR3-specific α-subunit of the CR3 heterodimer, thus confirming and extending our previous results.[28] We confirmed CR3 positivity of LC on skin sections by means of *in situ* IEM.[27] Previous immunohistological studies showed that 30% of dendritic EC were weakly reactive to the anti-CR3 α-subunit,[29] and that under culture conditions CR3 remained on LC.[30] However, we are the first to clearly show CR3 expression by virtually all human epidermal resting LC both *in situ*[27] and when freshly isolated.[28]

Failure to detect CR3$^+$ dendritic cells on sections of normal human skin may be due to the relatively few CR3 molecules present on LC of normal human epidermis. In this study, when methods of relatively low sensitivity were used, such as LM and FM immunostaining of frozen sections and of epidermal sheets, only faint staining of a few dendritic cells was observed. However, the IG-TEM method enabled detection of significant numbers of gold particles bound to the CR3$^+$ LC membrane, although less than seen on CD1a$^+$ control LC.[16,17,21,24] This relatively limited number of gold particles may be due to a weak CR3 expression by LC, since the number of antigenic sites may be correlated to the number of gold particles bound to the plasma membrane.[31] However, the density of gold granules (7·5 ± 9·5 particles per LC section) was sufficient to confirm the CR3 positivity of LC, and the IG-SEM method revealed a considerable number of gold granules over the entire surface of LC.

The conventional immunocytochemical methods which were unable to detect CR3 expression by LC also failed to reveal CD4 expression by LC in the same samples,[10,11] whilst more sensitive methods demonstrated CD4 positivity of LC.[16,21,32,33] The number of the CD4$^+$ LC detected rose as the sensitivity of the methods increased from one-step or two-step methods on cryostat sections, to a four-step method on cryostat sections, to epidermal sheets preparations, to *in situ* IEM.[32]

CR3 and CD4 positivity can be detected on HXC by methods that fail to show such positivity on LC cells.[10,11] Since histiocytosis X represents a proliferative disorder of LC origin, this may be due to an increase in CD4 and CR3 expression. CD4 expression by LC is weak in normal skin but increases in diseased skin,[32] and probably the degree of the CR3 expression by LC also alters according to conditions.

The precise role played by CR3 on the plasma membrane of LC is still unkown. However, since C3 receptor binds C3 sites of immune complexes, LC-bound antibody can mediate the presentation of antigen to T lymphocytes,[34] as do antigen-specific cell types, such as B cells by means of membrane-bound immunoglobulins.[35] Also complement receptors can modulate the interaction of LC with lymphocytes and other cell types.[36] The migration and the recruitment of LC to an inflammatory stimulus could be due to the CR3 expression on the plasma membrane, as demonstrated for the recruitment of monocytes.[37] CD1a$^+$ mononuclear cells resembling LC precursors and which showed migration[5-7] clearly expressed CR3. CR3 has been shown, when suitably ligated, to evoke various features of macrophage activation,[38] and CR3 may participate in the activation of LC by cytokines. Finally, LC are known to be phagocytic and CR3 is known to promote adherence of C3-opsonized particles to phagocytic cells.[39]

ACKNOWLEDGMENTS

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Cultured epithelia from junctional epidermolysis bullosa letalis keratinocytes express the main phenotypic characteristics of the disease

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SUMMARY

Keratinocytes from a 1-week-old male infant with junctional epidermolysis bullosa letalis (JEBL) were grown *in vitro* and then grafted as multi-layered epithelia onto nude mice, to investigate whether the defect in the dermo–epidermal cohesiveness in the disease is of epidermal and not mesodermal origin. In culture, there was a birefringent ring of cells at the edges of the keratinocyte colonies and in places some cells looked as though they had been ejected from the periphery of the colony. At confluence, the multi-layered epithelia were easily detached from the culture flasks using only mechanical agitation. On microscopy the fully-differentiated epithelium on days 21, 30 and 40 after grafting sometimes showed blistering at the dermal–epidermal junction. No labelling was noted using a GB3 monoclonal antibody, that reacts with normal human keratinocytes in culture and with the dermo–epidermal basement membrane zone in normal skin. This indicates that the defect of JEBL may be reproduced in culture and also after grafting the cultured epithelia onto a wound without an epidermis. This suggests a possible role for the junctional structure recognized by GB3 in dermo–epidermal cohesiveness.

Junctional epidermolysis bullosa letalis (JEBL) is a rare, severe genodermatosis characterized by a defect in the cohesiveness between connective tissue and epithelium in many tissues,[1–3] and especially the skin.[4–8] Blister formation occurs at the dermo–epidermal junction at the lamina lucida in relation to abnormal hemidesmosomes.[9–14] Recent studies have demonstrated that JEBL is usually correlated with an absence of reactivity using immunofluorescent techniques and a GB3 monoclonal antibody (mAb). This is an antibody that is directed towards a 600

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kilodalton (kDa) molecular weight protein in the lamina lucida.[15-19] In this study, keratinocytes from a child with JEBL were grown in culture and grafted onto nude mice to determine the reconstruction of the basement membrane zone (BMZ) after terminal differentiation of the grafted cells.

METHODS

Patient

A 1-week-old male Caucasian infant, the first child of a 20-year-old mother, following a normal pregnancy was admitted with severe blistering on the face and the hands. There was no previous family history of blistering and the parents were first cousins. The blisters were subepidermal (Fig. 1a) and electron miscroscopy confirmed the diagnosis of JEBL (Herlitz–Pearson type), showing blistering at the lamina lucida with abnormal hemidesmosomes and an absence of the sub-basal dense plates and there were rudimentary attachment plaques (Fig. 2).[14] Another criterion for JEBL was the absence of reactivity at the BMZ with monoclonal antibody GB3.[16] Other normal components of the BMZ such as type IV collagen, laminin, and bullous pemphigoid (BP) antigen were detected by indirect immunofluorescence (IF). No oesophageal atresia was detected, but continued blistering of the skin on the trunk, buttocks, arms and legs without healing, eventually led to the death of the child at the age of 8 months.

Epidermal cell culture

Human JEBL keratinocyte suspensions were prepared from suction-blister roof specimens using a standard trypsinization procedure. After centrifugation the cells were resuspended in medium as previously described[20] and then seeded onto 3T3 feeder layers.[21] At confluence the keratinocytes were trypsinized with a 1:1 mixture of trypsin/EDTA, then resuspended and

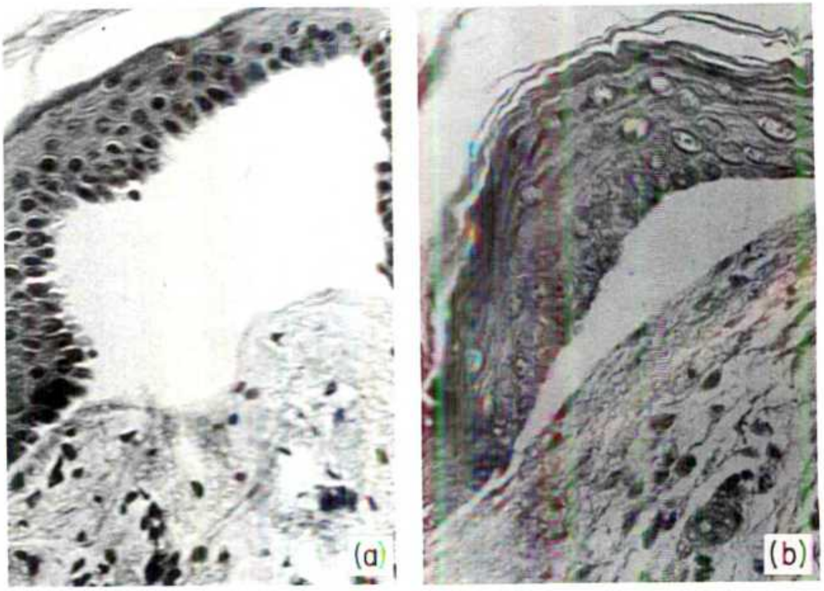

FIGURE 1. Subepidermal blisters. (a) Section of the patient's skin; (b) section of first-passage cultured epidermis after grafting on nude mouse (H. & E., original × 200).

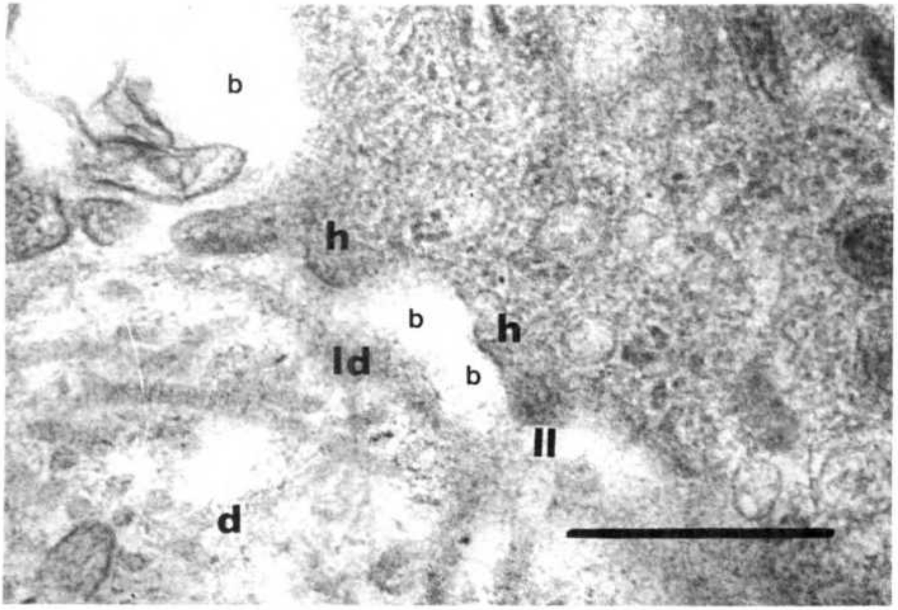

FIGURE 2. Electron microscopy supporting the clinical diagnosis of JEBL, and showing the rudimentary attachment plaques and the absence of sub-basal plates. h, hemidesmosomes; b, blisters; ld, lamina densa; ll, lamina lucida; d, dermis. Scale bar: 0·5 μm.

seeded onto 3T3 feeder layers. The cultures were incubated at 37°C in a 10% CO_2 and 100% humidity atmosphere and the medium changed every 2 days.

Control epidermal cell cultures were prepared from normal skin removed at plastic surgery from three adult donors.

Transplantation

The grafting method was a modification of that described by Banks-Schlegel and Green.[22] In each group 21 6-week-old congenitally athymic nude mice (Swiss Nu/Nu, SCCup, IFFA-CREDO, Lyon, France) were anaesthetized using intraperitoneal injection of sodium pentobarbital 0·4 mg/10 g body weight (Nembutal, Abbot, France). A 14-mm diameter circular graft bed was prepared on the dorso-lateral side of each animal with removal of full-thickness skin down to the fascia. Following detachment from the flask, the cultured epithelium on sterile gauze was placed in contact with the graft bed of the mouse and with the edges under the adjacent skin. A hat-shaped plastic capsule was placed over the gauze on the graft and fixed with four loose sutures to the surrounding mouse skin. Dressings were used to further secure the graft.[23] Ten days after grafting, the bandage was cut with scissors and gently removed with forceps. After inspection of the graft the site was rebandaged with several inspections carried out at various intervals.

Differentiation studies

The *in vitro* reconstituted epithelia from JEBL and from normal control keratinocytes were examined at confluence of the secondary cultures. Twenty-one, 30 and 40 days after grafting seven mice in the group were killed and after removal of the dressing, capsule and gauze, the grafted zone was then excised with the surrounding mouse skin to determine the junction between the grafted human epithelium and the underlying tissue. For routine histology, the samples were fixed in Boulin's fixative medium, dehydrated in alcohols, and embedded in paraffin. Thick sections were stained with haematoxylin–eosin–safran. For indirect IF studies, frozen sections were treated with the following antibodies: rabbit polyclonal antibody to type IV collagen, diluted 1/100 in PBS (Institut Pasteur, France); rabbit polyclonal antibody to laminin, diluted 1/100 in PBS (Institut Pasteur); serum from patients with bullous pemphigoid, diluted 1/100 in PBS; GB3, a mouse IgG 1 to a 600 kDa protein of the BMZ, unreactive on skin of patients with JEBL as previously described;[16,18,19] rabbit polyclonal antibody to involucrin (Biomedical Technologies Inc, Stoughton, WI, U.S.A.), used because of its human specificity.[23] In each case, the appropriate conjugate was used in the second step of the reaction. For electron microscopy (EM), specimens were fixed in 2% glutaraldehyde, post-fixed in osmium tetroxide, and embedded in epon resin. Semi-thin and ultra-thin sections were cut on a Reichert Om U 300 ultra-microtome, and sections stained with uranyl acetate and lead citrate and then examined in a Phillips EM 300 electron microscope.[24]

RESULTS

Cell culture observations

The plating efficiency was similar whether the cells were from normal or JEBL epidermis. Confluence was obtained in the primary cell culture within 10 days. We noted a reduced time when producing the cells in suspension with incubation using trypsin–EDTA in the JEBL cultures. This was 3 min as compared with 15 min for the controls.

In primary culture and in the subcultures, the colonies of JEBL cells seen, using an inverted microscope, looked somewhat abnormal with a birefringent ring at the periphery (Fig. 3a). This

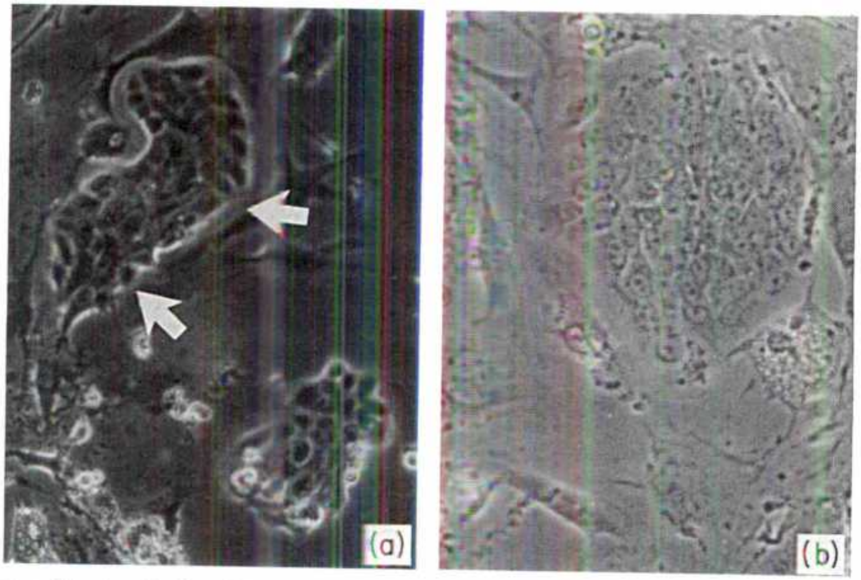

FIGURE 3. Colonies of cultured human keratinocytes from (a) JEBL epidermis and (b) control skin. Note the birefringent cells at the periphery of JEBL cell colonies (arrows) (× 200).

feature was observed as early as day 4 in the primary cultures and by day 2 in the subcultures and could be related to defective attachment of the cells to the culture flask. This was never noted with the control cells (Fig. 3b). Nevertheless JEBL cell confluence was obtained after 7 days in subculture.

The JEBL cell confluent subcultures showed detachment of the reconstituted epithelial sheets following only minimal mechanical agitation. This was never observed with the control cultures. Detachment of normal keratinocytes grown under similar conditions required incubation in dispase for 1 h.[25]

Epithelial sheet observation

No macroscopic differences could be observed between epithelia grown from JEBL cells and control epithelia.

On light microscopy, the JEBL-cultured epithelium showed the histological features of normal cultured human epidermis[20] and was composed of 5–8 layers of cells.

Using immunofluorescence the BP antigen was detected on JEBL epithelia as in the controls, whereas GB3 was negative on the JEBL specimens but positive in the controls.

On electron microscopy the JEBL-reconstituted epithelium was similar to that of normal epithelium. No hemidesmosomes were observed in the basal cells as usually seen with cultured epithelium.[24]

Grafted tissue studies

Macroscopically there was no significant difference between JEBL grafts and the controls. Less grafts took in the JEBL group, 15/21 as compared with 19/21 for the controls. No blisters were noted on the JEBL epithelial grafts and the Nikolski sign was negative.

On light microscopy a fully differentiated epidermis was observed as early as 21 days following grafting. The dermo–epidermal junction was linear, and no inflammatory changes were observed in the dermis. The findings were similar to those seen in the normal epithelium grafted onto nude mice. A lack of adhesion between the epithelium and the underlying connective tissue was noted in the JEBL group, with a longitudinal subepidermal split of approximately half the length of the grafts. True subepidermal blisters, with a roof constituted of a normal-looking epidermis, were noted, with features similar to those observed in the patient (Fig. 1b).

On immunofluorescence the JEBL grafts were unreactive when tested with the GB3 antibody, whereas a linear fluorescent pattern was noted at the dermo–epidermal junction of epithelia derived from normal keratinocytes and grafted onto the nude mice (Fig. 4). Laminin, type IV collagen and BP antigen could be detected at the normal location in both JEBL grafts and in the controls. The grafted epithelia expressed involucrin, which shows the viability of human epithelium at 21, 30 and 40 days after grafting and its non-replacement by murine epidermal cells.

Ultrastructural analysis showed a terminal differentiation of the grafted epidermis after 30 days. Some lipid inclusions were found in the horny layer. The keratohyalin granules appeared normal in size and number and membrane-coating granules were also found. The tonofilaments, cell-junctions and desmosomes appeared normal. The basal cells of JEBL-grafted epidermis were vacuolated, and unlike the reconstituted normal epidermis, showed a lack of hemidesmosomes, lamina densa and anchoring fibrils. An obvious separation was observed between the basal layer and the underlying dermis (Figs 5 and 6).

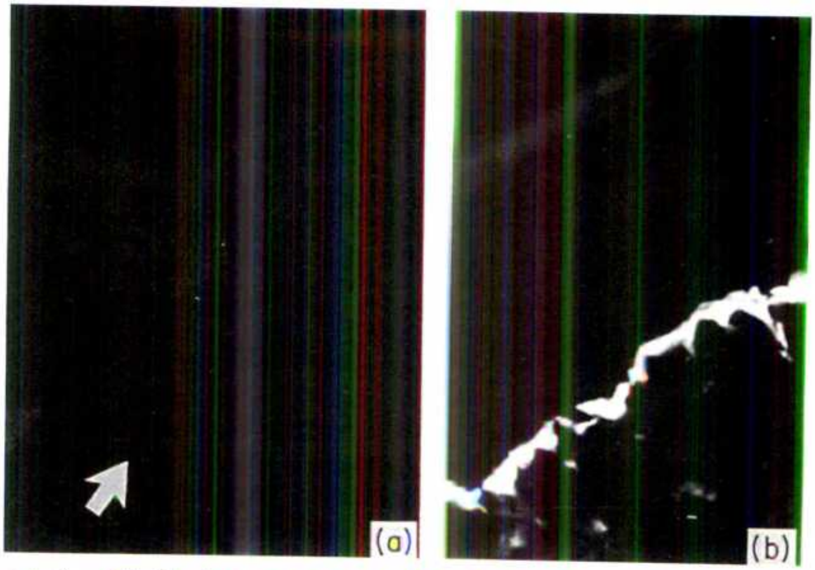

FIGURE 4. Indirect IF of the junction between grafted cultured epithelia and the murine wound bed, reactivity with the mAb GB3. There is an absence of reactivity with (a) JEBL epithelium and a normal linear pattern with (b) control human cultured epidermal sheet at the dermo–epidemal junction (arrow, × 400).

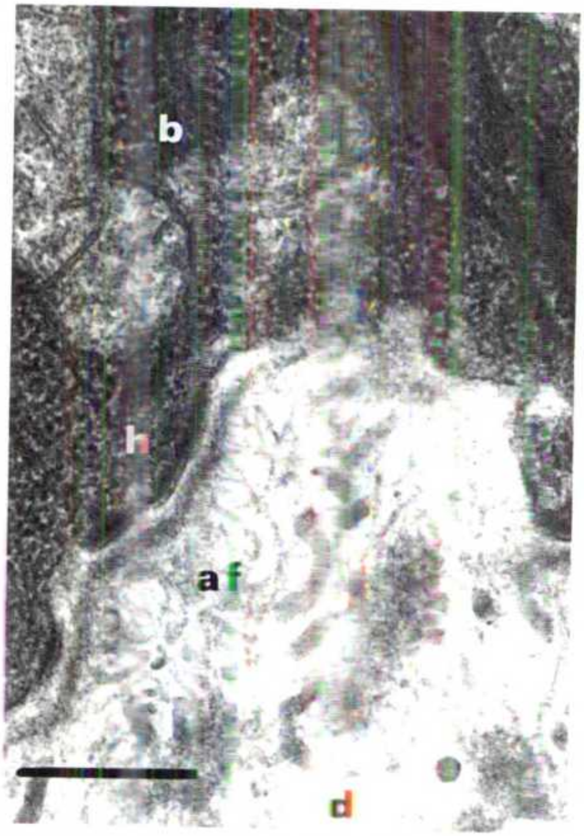

FIGURE 5. Electron microscopy of a section of Day 30 epidermal sheet from control human keratinocytes grafted on nude mouse dermis. (b, basal keratinocytes; h, hemidesmosome; af, anchoring fibril; d, dermis; scale bar 0·375 μm).

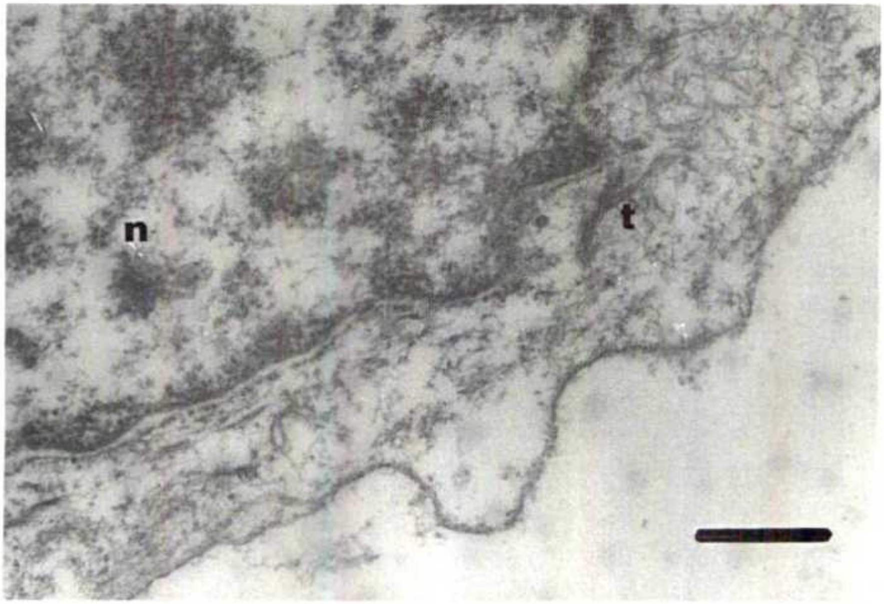

FIGURE 6. Electron microscopy of JEBL cultured epidermis 30 days after grafting: subepidermal blister with absence of hemidesmosome (n, nucleus; t, tonofilaments; scale bar, 0·5 μm).

DISCUSSION

Our studies have shown that keratinocytes from JEBL epidermis may be grown *in vitro* into multi-layered epithelia. These epithelia exhibit both in culture and after grafting onto surgical wounds several features that suggest an epithelial origin for the lack of dermo–epidermal cohesiveness characteristic of this disease.

Human epidermal cells can be grown on 3T3 cell-feeder layers into multi-layered epithelia, though differentiation is only fully achieved following grafting onto dermal human wounds[20,24] or surgical wounds, as in the nude mouse model.[22,23] Under these conditions a dermo–epidermal junction, with the presence of type IV collagen, laminin, BP antigen (by IF) and hemidesmosomes (by EM), a lamina densa and anchoring fibrils, is usually reconstituted within a few weeks after grafting.[20,22,24]

In this study, keratinocytes from a patient with JEBL were seeded onto 3T3 cells, and formed colonies that allowed the formation of a multi-layered epithelium suitable for grafting and with no abnormal *in vitro* differentiation.[20] Evidence of a defective cohesiveness of the cultured cells to their substrate was noted. Cultured keratinocytes from the primary culture were resuspended after a 3-fold reduced incubation time with trypsin–EDTA solution. Cell colonies were surrounded by a peripheral ring of birefringent cultured cells, not noted in the controls. While dispase treatment is necessary to detach an intact epithelial sheet from its support, no enzymatic treatment was necessary with the JEBL epithelia. Previous studies[26] have shown the spontaneous formation of blisters *in vitro* with cultures of JEBL keratinocytes after 14–22 days. However, in our studies we did not observe similar blisters. This may have been due to the shortened time needed for cells to reach confluence in our system (7 days in subculture). The reduced efficiency of grafting may reflect the detachment of JEBL cells *in vitro*. On examination of the grafted epithelium, a clear dermo–epidermal separation was noted with the formation of subepidermal blisters. Ultrastructural observations contrasted with previous studies of the reconstitution of the BMZ following the grafts of normal cultured human epithelia.[20,22,24]

The epithelia did not express the antigenic determinant as recognized by the MAb GB3 which is an anti-amnion antibody and reacts with several epithelial–connective tissue junctions.[18] In the skin, this IgG 1 antibody is directed towards a 600 kDa protein in the lamina lucida. In culture, normal human keratinocytes exhibit a positive intracellular staining with GB3.[19] A defective reactivity with GB3 was shown in JEBL skin and GB3 has been proposed for the prenatal diagnosis of this genodermatosis.[15] In our study, cells from JEBL epidermis did not express the protein detected by GB3. It was also absent after JEBL epithelia were transplanted, in contrast with the reactivity with GB3 at the BMZ when normal reconstructed epithelia was used for grafting. This is further evidence that the antigen as detected by GB3 has a role in the pathogenesis of JEBL.

Finally, JEBL cells were grown *in vitro* in the absence of any human mesenchymal influence from the original diseased skin, indicating that this genetic disorder is epithelial in origin. The possibility of additional defects such as abnormalities of fibroblasts however cannot be excluded. This model of lesional keratinocyte cultures, followed by grafting onto a living substrate in order to achieve a full differentiation, has been used to prove the ectodermal origin of such disorders of keratinization as lamellar ichthyosis.[27] A similar model represented by cell cultures from basal cell suspensions grafted onto a healthy dermis has been used to reproduce the epidermal lesions of Hailey–Hailey disease.[28,29]

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A dose-response study of UVB-induced suppression of contact photosensitivity in the mouse

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SUMMARY

A marked contact photosensitivity (CPS) response to 3,3′,4′,5 tetrachlorosalicylanilide (TCSA) plus UVA was induced in mice. Cyclophosphamide (Cy), given prior to sensitization, caused a further increase in ear swelling. When UVB radiation was given at a site distant from that used for sensitization it caused a dose-related suppression of CPS. Cy did not eliminate the UVB-induced suppression.

There has been considerable interest in the deleterious effects of ultraviolet radiation (UVR), in particular UVB (290–320 nm), on the immune system.[1,2] These effects include a reduction in the ability of UVB-irradiated mice to reject highly antigenic UV-induced tumours,[3,4] and a reduction in the intensity of contact sensitivity (CS)[5] and contact photosensitivity (CPS) reactions.[6,7] UVB has been shown to have numerous effects on epidermal cells. It reduces the number and function of Langerhans cells[8] and causes the release of cytokines[9] and other soluble factors[10] from damaged epidermal cells.

When UVB is given at a site distant from that used for contact sensitization in mice, it leads to changes in the distribution and function of antigen presenting cells in the spleen[11,12] and changes in the distribution of activated lymphocytes in the lymph nodes.[13] In addition, hapten-specific T-suppressor cells (Ts) have been found in the spleens of mice exposed to UVB before sensitization with contact allergens.[14]

We have studied the effects of broad-band UVB on CPS to 3,3′,4′,5 tetrachlorosalicylanilide (TCSA) in mice, with the UVB given at a site distant from that used for sensitization. The objectives of the study were: to determine whether UVB-induced suppression of CPS was dependent on UVB dose, and to determine whether UVB-induced suppression of CPS is mediated by a cyclophosphamide (Cy) -sensitive mechanism.

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METHODS

Animals

Female BALB/cJ mice, 8–10 weeks old, were obtained from Jackson Laboratories, Bar Harbor, ME, U.S.A. and housed in a light-cycled room. The treatment groups consisted of a minimum of nine to a maximum of 20 animals.

UVA and UVB sources

UVA radiation was provided by two General Electric F20T12 BL fluorescent lamps (G.E.Canada). A 3·15 mm thick sheet of glass was used to exclude UVB radiation. UVA irradiance was measured with an IL570 International Light meter, SEE 010 # 750 detector and UVA # 339 filter, with half power points 328–376 nm (International Light Inc., Newburyport, MA, U.S.A.). For UVA irradiation, the mice were placed in compartmentalized wire cages above the lamps and the radiant intensity was 0·6 mW/cm^2.

The UVB source consisted of two FS20T12 fluorescent lamps (National Biologic Corp., Twinsburg, OH, U.S.A.). UVB irradiance was measured with the IL570 light meter, SEE 240 # 728 detector and filter # 68, with half power points 275–303 nm. For UVB irradiation, the mice were placed below the lamps and covered with a wire mesh screen. The radiant intensity with the wire mesh screen was 0·11 mW/cm^2.

Reagents

TCSA was obtained from Eastman Kodak, Rochester, NY, U.S.A. Cyclophosphamide (Cy) was purchased from Procytox, Montreal, Canada.

Treatments

Sensitization and challenge. Mice were anaesthetized with ethyl ether and 50 µl of 1·0% TCSA in acetone was applied to a clipped area on the ventral surface. The skin on the ventral surface was then exposed to a UVA dose of 3·0 J/cm^2 (day 0). This procedure was repeated the next day (day 1). On day 7, the mice were challenged on the ears with 10 µl of 0·5% TCSA and exposed to 6·0 J/cm^2 of UVA. Ear measurements were taken before challenge and at 24-h intervals thereafter for 3 days.

UVB irradiation. Two days before sensitization (day − 2), groups of mice were anaesthetized, and hair was clipped from a dorsal area approximately 2 by 2 cm. Ears were protected from UVB with black electrical tape. The groups of mice were then irradiated with either 10, 20, 40, 100, 300, 500 or 1000 mJ/cm^2 of UVB.

Cyclophosphamide. Groups of mice were injected intraperitoneally with a dose of 200 mg/kg body weight of Cy in sterile distilled water, 3 days before UVB irradiation (day − 5).

Controls. One group received no Cy or UVB, but underwent the normal sensitization and challenge protocol. A second group received no UVB, but received Cy 5 days before sensitization, and also underwent challenge. A third non-sensitized group received Cy 12 days before the standard challenge procedure and a fourth group received the challenge procedure alone. The third and fourth groups were phototoxic controls.

Per cent suppression.

The per cent suppression induced by UVB radiation is calculated using the following equation:

$$\% \text{ Suppression} = 1 - \left(\frac{A-B}{C-B}\right) \times 100,$$

where A is the ear swelling of the group irradiated with UVB before sensitization, B is the ear swelling of the non-sensitized (negative control) mice and C is the ear swelling of the control group sensitized without UVB irradiation.

Statistical methods

The numerical results reported are mean ± standard error of mean of 24 h results. Net photosensitivity responses are given as the total ear swelling minus the phototoxic control value. The statistical analysis for comparison of means was by the Student's *t*-test and ANOVA test.

RESULTS

Standard sensitization

The standard sensitization procedure showed an ear swelling response of $16.0 \pm 0.7 \times 10^{-3}$ cm. Cy given before sensitization resulted in a significant increase to $22.2 \pm 1.3 \times 10^{-3}$ cm ($P < 0.0005$).

UVB-induced systemic immune suppression

UVB irradiation of the back, 2 days before sensitization on the abdomen, reduced the intensity of the subsequent ear swelling reaction in a dose-dependent manner (Fig. 1). UVB doses of 10 and 20 mJ/cm² produced no significant change in ear swelling response from the control ($P > 0.05$). However, with UVB doses of 40, 100, 300, 500 and 1000 mJ/cm² there were significant dose-related decreases in CPS responses ($P < 0.05$) when compared to control. In addition this suppression is shown to be linearly related to the $\log_{10}$ UVB dose (Fig. 2).

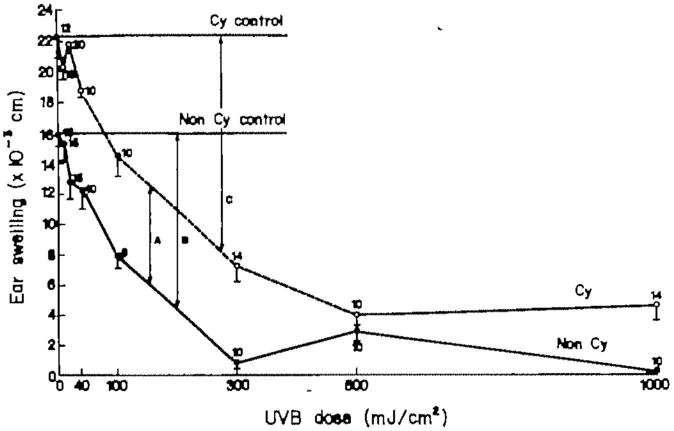

FIGURE 1. Ear swelling measurements as a measure of the suppressive effect of various doses of UVB on CPS in mice with or without prior Cy treatment. Shown are the mean increases ± standard error of means. The number of animals for each data point is given. A, Suppression induced at sensitization; B and C, suppression induced by UVB.

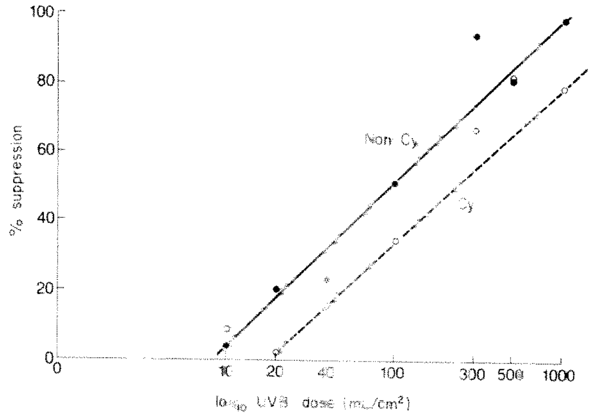

FIGURE 2. Relationship of log of UVB dose to the suppression of CPS to TCSA in mice with and without prior Cy treatment.

Effects of cyclophosphamide on UVB-induced systemic immune suppression

In the Cy treated groups, significant UVB-induced suppression was again first detected with a dose of 40 mJ/cm^2 ($P < 0{\cdot}025$). Suppression increased with increasing doses of UVB (Fig. 1) and is linearly related to the $\log_{10}$ UVB dose (Fig. 2). Thus, UVB-induced suppression was not prevented by prior treatment with Cy (Fig. 1), and the Cy effect was constant over the whole dose range of UVB tested (Fig. 2).

Statistical analysis

Student's *t*-test was used to identify the lowest UVB dose to induce significant suppression, when compared to the non-UVB irradiated control. In both Cy treated and non-Cy treated groups of mice, the first effective UVB dose to induce significant suppression was 40 mJ/cm^2.

The ANOVA test showed that there was no interaction between Cy and UVB, that UVB induced a dose-related suppression of CPS, and that Cy enhanced CPS responses at all UVB doses.

DISCUSSION

Contact photosensitivity (CPS) is a form of delayed-type hypersensitivity reaction similar to contact sensitivity (CS) except that UVA is required at sensitization and challenge.[7,15] Since we do not employ UVB,[15] Cy[16], or other immunological adjuvants[17] in our standard sensitization procedure we have a less complex model for studying factors that may modulate CPS. However, when we add Cy before sensitization the response is enhanced by almost 30%. Since Cy is believed to inhibit the development of Ts cells,[18,19] our observations confirm those of others who have demonstrated that Cy-sensitive Ts cells are generated at sensitization with TCSA.[16]

This is the first study to show a dose-response curve for the systemic effect of broad band UVB on CPS, i.e. when UVB is given to a site distant from that used for sensitization. In our study, UVB-induced immune suppression was first detected with a dose of 40 mJ/cm^2 and suppression increased with higher doses. The immune suppression is also shown to be related linearly to $\log_{10}$ of the UVB dose for the UVB doses tested (Fig. 2). A 50% suppression of CPS was seen with approximately 100 mJ/cm^2 of UVB. The threshold dose of 40 mJ/cm^2 is considerably lower than the single dose of 2500 mJ/cm^2 given by Granstein *et al.*,[16] who did not determine the threshold dose. Caution is required when comparing UVB doses from different studies due to the different techniques and equipment used for measuring radiation, but we feel it is necessary to comment on other findings despite the lack of standardization in UV measurements.

Miyachi *et al.*[20] were unable to demonstrate systemic suppression of CPS to TCSA in a ICR mouse strain with a dose of 40 mJ/cm^2 of UVB. This may be due to the fact that their TCSA sensitization procedure produces lower reactions. In addition, they used ICR mice while we used BALB/cJ, and we have shown a considerable variation in the degree of CPS reactivity among eight different mouse strains.[21] Granstein *et al.*,[16] using cell transfer experiments, were unable to demonstrate Ts cells in mice in which CPS to TCSA had been systemically suppressed by UVB. However, they use Cy 3 days after UVB as an essential part of their sensitization protocol. Their results led them to suggest that a second form of anergy, not mediated by suppressor cells, had been produced by UVB in these animals.

In the present system we have shown two different suppressive components. The first is an integral part of the normal sensitization process and it is sensitive to Cy treatment. This suppression may be related to the dose of antigen used at sensitization.[22] The second is that induced by exposure to UVB, which is not sensitive to Cy. Thus, it seems unlikely that Cy-sensitive Ts play a major role in UVB-induced systemic suppression of CPS. Our results with Cy show that UVB does not produce its systemic suppressive effect by inducing Cy-sensitive Ts. We suggest that there may be some other mechanism as proposed by Granstein *et al.*[16] This is contrary to the suggestion of Kripke,[23,24] who proposed that systemic suppression of CS by UVB is due to antigen-specific T-suppressor cells. However, our results show that if such UVB-induced suppressor cells exist in CPS they are not Cy-sensitive. It is possible that there are different mechanisms for UVB suppression of CPS and CS but these immune reactions are considered to be similar.[25] An alternative view of the mechanism of UVB suppression of CS suggests that UVB given before induction may produce trapping of circulating effector cells within peripheral lymph nodes resulting in suppression.[26,27] It is known that a suppressor mechanism is activated at sensitization and we have shown a similar process with CPS to TCSA but the elimination of this suppression with Cy does not prevent the UVB-induced suppression.

Although we have not found the mechanism by which UVB-induces its suppression, we have shown that it is not Cy-sensitive, and that this suppression is linearly related to the $\log_{10}$ dose of UVB and can be achieved with low doses of UVB.

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Pro-inflammatory mediators induce sustained release of prostaglandin E_2 from human dermal microvascular endothelial cells

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SUMMARY

The vasodilator prostaglandin E_2 has been proposed as a mediator of erythema in a variety of cutaneous inflammatory reactions and prostacyclin levels have been found to be elevated in ultraviolet induced erythema. Human recombinant interleukin 1α and lipopolysaccharide induced a concentration- and time-dependent release of prostaglandin E_2, but not prostacyclin, from cultured neonatal and adult human dermal microvascular endothelial cells. Prostaglandin E_2 was measurable at 2 h after stimulation with 1 U/ml interleukin 1α, levels increased rapidly up to 6 h and more slowly up to 24 h. Lipopolysaccharide (20 μg/ml) induced measurable release of prostaglandin E_2 between 2 and 4 h after stimulation and release continued up to 24 h when incubation was terminated. With both agonists, release of prostaglandin E_2 was inhibited by indomethacin and significantly reduced by cycloheximide. The sensitivity and magnitude of responses of the cutaneous endothelial cells to these pro-inflammatory stimuli appeared to be dependent on their derivation.

There is substantial evidence that human skin is a source of pro-inflammatory molecules including eicosanoids, platelet-activating factor and the cytokine interleukin 1 (IL-1). IL-1-like activity can be extracted from the epidermis[1] and is released from normal human skin immediately following abrasion.[2] Exposure to UVB irradiation augments the release of IL-1-like activity.[3] The pro-inflammatory nature of IL-1 has been demonstrated in human skin by intradermal injection of human recombinant IL-1α (hrIL-1α), which induced a dose-related

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erythema, maximal at 24 h and persisting up to 48 h. In the early stages, the erythema was associated with oedema and a mixed cellular perivascular infiltrate.[4] These *in vivo* observations show that the dermal microvasculature is involved in the cutaneous inflammatory response to IL-1, but the delayed onset and persistence of the erythema indicate that other pro-inflammatory molecules, possibly released from the dermal microvasculature, may be involved.

Both IL-1 and bacterial endotoxin have been shown to elicit similar responses in human endothelial cells cultured from umbilical and saphenous veins. These include release of prostacyclin (PGI_2),[5] the potentiation of neutrophil and monocyte adherence[6] and expression of specific surface antigens.[7] The structure/function relationships of vascular endothelium are influenced by its environment, so that data obtained from experiments employing large-vessel endothelial cells cannot necessarily be extrapolated to the microvasculature, that is primarily involved in inflammatory processes. It has been demonstrated that dermal microvascular endothelial cells derived from neonatal foreskin release more prostaglandin E_2 (PGE_2) than PGI_2 when stimulated with the calcium ionophore A23187,[8] but the requirements for PGE_2 release were not examined.

We have cultured microvascular endothelial cells from the dermis of both neonatal foreskin and adult facial skin, characterized them using immunocytochemical methods and investigated whether these cutaneous microvascular endothelial cells can release vasoactive prostaglandins in response to the pro-inflammatory stimuli IL-1 and bacterial endotoxin.

METHODS

Materials

Culture medium and antibiotics were obtained from Gibco, Paisley, Scotland, U.K. Additional cell culture supplements, lipopolysaccharide from *E. coli* (serotypes 055: B5 and 0127: B8), cycloheximide, actinomycin D, tunicamycin, indomethacin, trifluoperazine and *Ulex europaeus* agglutinin 1 were obtained from Sigma Chemical Co., Poole, Dorset, U.K.

All antibodies, except the endothelium-specific monoclonal antibodies (mAb), were obtained from Dakopatts, High Wycombe, U.K. Of the mAbs, PAL-E was a gift from Dr R.O.Schlingemann, The University of Leiden, The Netherlands, EN 4 was a gift from Dr C.J.F.Spry, The Royal Postgraduate Medical School, London, U.K. and BMA 120 was obtained from Behring, Marburg, F.R.G.

The 6-keto-prostaglandin $F_{1\alpha}$ antiserum was a gift from Dr M.Greaves, Department of Haematology, Royal Hallamshire Hospital, Sheffield, U.K. The PGE_2 antiserum and the prostaglandin standards were obtained from Cayman Chemicals, Ann Arbor, MI, U.S.A. and the tritiated prostaglandins were obtained from Amersham PLC, Little Chalfont, U.K.

Human recombinant interleukin 1α was a gift from Dr P Lomedico, Hoffman La Roche Inc., Nutley, NJ, U.S.A. This preparation consisted of the carboxy-terminal 154 amino acids of the 271 amino acid human IL-1α precursor and was essentially pure protein with specific activity of $1{\cdot}3 \times 10^6$ half maximal U/ml, 5×10^6 half maximal U/mg protein and contained less than 3×10^{-5} EU/ml endotoxin, measured by the Limulus assay.

Endothelial cell cultures

Human dermal microvascular endothelial cells were isolated from both neonatal foreskin obtained after routine circumcision and adult facial skin discarded after elective cosmetic surgery and established in culture using a modified method of Davison and Karasek.[9,10]

The foreskins were cut open and all underlying connective tissue removed. After being extensively washed in calcium- and magnesium-free Hanks' balanced salt solution (HBSS) containing 200 U/ml penicillin, 200 μg/ml streptomycin, 100 μg/ml amphoteracin B and 100 g/ml kanamycin, the pieces of skin were placed in a solution of 0·5% trypsin prepared in isotonic saline (140 mM NaCl, 6 mM KCl, 4 mM glucose, pH 7·2) and incubated overnight at 4°C.

The sections of facial skin were washed in HBSS plus antibiotics and all underlying fat removed. The epidermis and papillary dermis were separated from the underlying dermis using a Castroviejo keratotome set to cut at 0·3 mm. These sections of skin were washed in HBSS containing antibiotics and then incubated overnight at 4°C in 0·5% trypsin.

The next morning, the skin sections from either source were washed twice in HBSS and the epidermis removed. The dermal sections were then washed extensively in HBSS and transferred individually to a Petri dish containing a small volume of Iscove's modification of Dulbecco's modified Eagle's medium (DMEM). The capillary segments were expressed into the culture medium, centrifuged at 600 ***g*** for 2 min, resuspended in culture medium and centrifuged again. The cells were resuspended in complete culture medium consisting of Iscove's DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml amphoteracin B and supplemented with 10^{-4} M hypoxanthine, 10^{-3} M oxaloacetic acid, 4×10^{-4} M pyruvate, 25 IU/100 ml insulin, $1{\cdot}6 \times 10^{-5}$ M thymidine, $3{\cdot}3 \times 10^{-5}$ M isobutylmethylxanthine (IBMX), 5×10^{-4} M dibutyryl cyclic AMP (cAMP) and 30% pooled human serum (PHS). They were plated onto Petri dishes previously coated with human fibronectin prepared from cryoprecipitate.[11]

The cells used in these experiments were confluent monolayers of passages 2 to 4.

Identification of human dermal microvascular endothelial cells in culture

The endothelial cell nature of the cultured dermal cells was determined by immunocytochemical staining for von Willebrand factor protein (vWF)[12], binding of the lectin *Ulex europaeus* agglutinin-1 (UEA)[13] and labelling by the endothelium-specific mAbs EN4[14], PAL-E[15] and BMA 120.[16]

Confluent monolayers of human dermal microvascular endothelial cells were washed twice with culture medium alone and overlayed with 1 ml of fresh medium. The cells were scraped gently from the plates into the culture medium and cytocentrifuged onto glass slides (Shandon cytospin 2 centrifuge, 10 min, 100 ***g***). The cells were harvested mechanically so as to avoid the use of trypsin which might have destroyed labile cell-surface antigenic sites. The cytospin preparations were fixed immediately in 70% methanol at 4°C for 20 min, air-dried and processed.

Staining procedures

UEA and all antibodies were diluted in phosphate-buffered saline (140 mM NaCl, 150 mM PO_4, pH 7·2) containing 1% w/v bovine serum albumin (PBS–BSA). Rabbit polyclonal antibody to UEA and mAb to vWF were diluted 1:100. The mAbs PAL-E, EN 4 and BMA 120 were used undiluted. Fluorescein isothiocyanate (FITC)-conjugated swine anti-rabbit IgG was diluted 1:150 and FITC-conjugated rabbit anti-mouse IgG was diluted 1:100.

The fixed cytospin preparations were washed with PBS. Those cell preparations to be used for assessment of UEA binding were incubated with UEA (0·002% w/v in PBS–BSA) for 2 h prior to treatment with the primary anti-UEA antibody. All cell preparations were incubated with the different primary antibodies overnight in a humidified chamber to facilitate maximum binding. They were then washed and overlayed with the FITC-conjugated second antibody for

30 min, washed again and mounted in glycerol/PBS. The mounted slides were viewed using a Nikon Diaphot microscope with epifluorescence filters.

Release of prostaglandins from endothelial cells

Prior to the performance of these experiments, human dermal microvascular endothelial cells were cultured for 24 h in Iscove's DMEM containing 30% PHS and all of the previously listed supplements except IBMX and cAMP. The experiments were carried out in serum free Iscove's DMEM containing all supplements except IBMX and cAMP.

Confluent monolayers of endothelial cells were washed once with serum-free culture medium and then overlayed with medium containing either human recombinant interleukin 1α (hrIL-1α) at concentrations between 0·01 and 5 U/ml or lipopolysaccharide (LPS) at concentrations between 0·1 and 50 μg/ml. The cells were incubated at 37°C for 24 h, after which time the supernatants were removed and frozen at −20°C for subsequent radioimmunoassay of 6-keto-prostaglandin $F_{1\alpha}$ (6keto-PGF$_{1\alpha}$) and prostaglandin E_2 (PGE$_2$). The endothelial cells were trypsinized (0·25 w/v trypsin in HBSS) and counted manually using an improved Neubauer Chamber (Weber, Lancing, W. Sussex, U.K.). Concentrations of prostaglandins were expressed in pg/10^4 cells.

The time course of release of prostaglandins was determined by incubating the endothelial cells with either 1·0 U/ml hrIL-1α or 20 μg/ml LPS. At different time points between 30 min and 24 h, the supernatant was removed and frozen at −20°C for subsequent radioimmunoassay for 6keto-PGF$_{1\alpha}$ and PGE$_2$.

The effect of specific antagonists on release of prostaglandins from endothelial cells

The concentrations of antagonists used in these experiments were not cytotoxic. They had previously been shown to induce neither morphological change, as assessed by phase contrast microscopy and trypan blue exclusion, nor direct stimulation of prostaglandin release following 24 h incubation with cells alone.

Confluent monolayers of endothelial cells were washed with serum-free medium and overlayed with serum-free medium containing the different antagonists. Following 30 min incubation at 37°C, 1 U/ml hrIL-1α or 20 μg/ml LPS was added and the cells incubated for a further 6 h at 37°C, at which time the supernatant was removed and frozen at −20°C for subsequent radioimmunoassay for 6keto-PGF$_{1\alpha}$ and PGE$_2$.

Prostaglandin assays.

PGI$_2$ and PGE$_2$ were measured using radioimmunoassays for 6keto-PGF$_{1\alpha}$ (the stable hydrolysis product of PGI$_2$) and PGE$_2$ respectively, as previously described.[17] The 6keto-PGF$_{1\alpha}$ antiserum exhibited cross-reactivity of less than 1% with other major prostaglandins of the 1 and 2 series and the limit of sensitivity of the assay was 50 pg/ml. The PGE$_2$ antiserum showed cross-reactivity of less than 1% with other prostaglandins of the two series and the limit of sensitivity of the assay was 150 pg/ml.

The cell supernatants were assayed without prior extraction and measurements made at two dilutions in each radioimmunoassay.

Analysis of data

Statistical analysis of endothelial cell responses to either hrIL-1α or LPS was performed using the Mann–Whitney *U*-test and results were considered to be significant if $P < 0·05$.

The effect of the metabolic inhibitors was analysed using paired Student's *t*-test and $P < 0·05$ was considered to be significant.

RESULTS

Identification of endothelial cells in culture

The neonatal and adult dermal microvascular endothelial cells showed a closely apposed cobblestone morphology (Figs 1a and e), the presence of vWF protein (Figs 1b and f) and binding of the lectin *Ulex europaeus* agglutinin-1 (Figs 1c and g).

Of the endothelium-specific mAb, only EN4 gave consistent positive staining of both neonatal and adult dermal microvascular endothelial cells (Figs 1d and h). Neither PAL-E nor BMA 120 bound to neonatal dermal microvascular endothelial cells. Some preparations of adult cells gave positive staining with BMA 120, whereas others did not and a few cells within individual preparations of adult cells exhibited weak positive-membrane staining with PAL-E (not shown).

Release of prostaglandins

In dermal microvascular endothelial cells incubated for 24 h with hrIL-1α, a concentration-dependent release of PGE_2 was observed (Fig. 2). A significant increase ($P < 0{\cdot}05$) in release of PGE_2 from both adult and neonatal cells was induced by 0·5 U/ml hrIL-1α. The neonatal cells were maximally stimulated with 1 U/ml hrIL-1α, but increasing amounts of PGE_2 were released from adult cells incubated with up to 5·0 U/ml. With the exception of the lowest concentration of hrIL-1α (0·01 U/ml), the neonatal cells released more PGE_2 than the adult cells exposed to the same concentration of agonist.

Release of PGE_2 from dermal microvascular endothelial cells incubated with hrIL-1α was time dependent (Fig. 3). In both neonatal and adult cells, incubated with 1 U/ml hrIL-1α levels of PGE_2 increased rapidly between 2 and 6 h following stimulation and thereafter increased more gradually up to 24 h when the incubations were terminated. The neonatal cells were more responsive to the agonist and released significantly more PGE_2 than the adult cells ($P < 0{\cdot}05$). 6keto-$PGF_{1\alpha}$ was undetectable in the supernatant of either adult or neonatal cells incubated with up to 5·0 U/ml hrIL-1α for 24 h.

Lipopolysaccharide (LPS) also induced a concentration-dependent release of PGE_2 from both adult and neonatal cells (Fig. 4). Release of PGE_2 from adult cells was significantly increased with 5 μg/ml LPS ($P < 0{\cdot}05$) and maximally induced with 20 μg/ml LPS. In contrast, 0·1 μg/ml LPS induced a significant increase in PGE_2 release from neonatal cells ($P < 0{\cdot}05$) and increasing amounts of PGE_2 were released from cells incubated with up to 50 μg/ml LPS.

Release of PGE_2 from both adult and neonatal cells incubated with 20 μg/ml LPS was time dependent (Fig. 5), with increasing release of PGE_2 occurring up to 24 h when the incubations were terminated. The neonatal cells were more responsive to the stimulus, PGE_2 being easily detectable in the cell supernatant at 4 h, when it was only just detectable in the supernatant of adult cells. Furthermore, after 24 h incubation, the amount of PGE_2 released from neonatal cells was significantly greater than that from adult cells ($P < 0{\cdot}05$). 6keto-$PGF_{1\alpha}$ was undetectable in the supernatant of either adult or neonatal cells incubated with up to 50 μg/ml LPS for 24 h.

In an attempt to determine the metabolic requirements for release of PGE_2 from human dermal microvascular endothelial cells incubated with hrIL-1α or LPS, confluent monolayers of cells were incubated with either indomethacin (cyclo-oxygenase inhibitor), actinomycin D (RNA transcription inhibitor), cycloheximide (protein translation inhibitor), tunicamycin (post translation protein glycosylation inhibitor) or trifluoperazine (calcium/calmodulin binding inhibitor). The hrIL-1α-induced release of PGE_2 from both adult and neonatal cells was inhibited by indomethacin and significantly reduced by cycloheximide ($P < 0{\cdot}005$). In adult

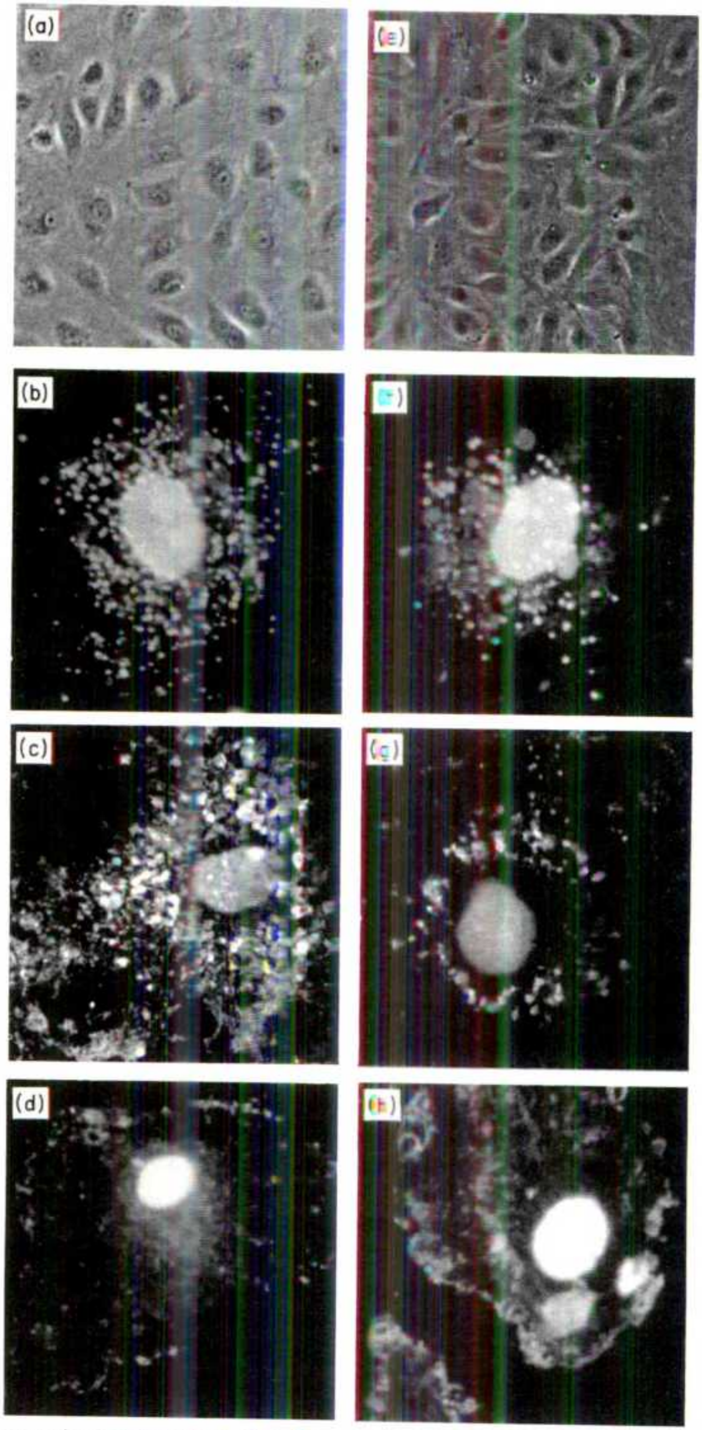

FIGURE 1. Characterization of adult (a–d) and neonatal (e–h) dermal microvascular endothelial cells. Phase contrast photomicrograph (a, e) (×200) and indirect immunofluorescent staining for von Willebrand factor protein (b, f), *Ulex europaeus* agglutinin-1 (c, g) and the endothelium specific mAb, EN 4 (d, h) (×400).

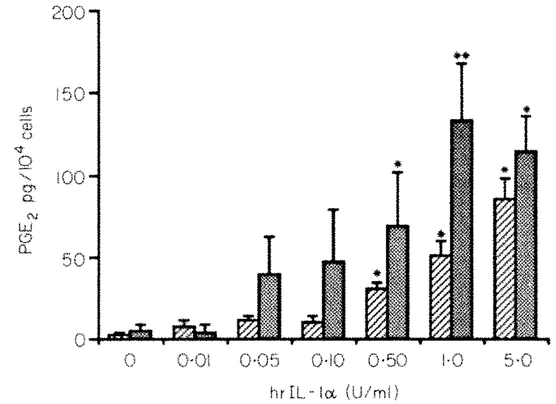

FIGURE 2. Release of PGE_2 from adult (■) and neonatal (▨) human dermal microvascular endothelial cells following 24 h incubation with concentrations of hrIL-1α between 0·01 and 5·0 U/ml. The columns represent the mean release of PGE_2 and the bars indicate the standard error of the mean (SEM) from six experiments performed using cells cultured from different donor tissue. $^*P < 0·05$, $^{**}P < 0·005$.

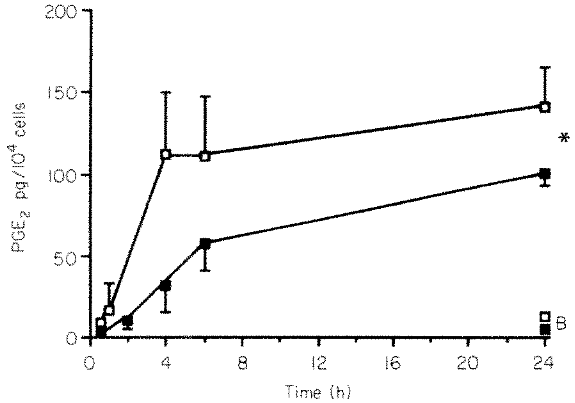

FIGURE 3. The time course of release of PGE_2 from adult (■) and neonatal (□) human dermal microvascular endothelial cells incubated with 1 U/ml hrIL-1α. B is the basal release of PGE_2 from unstimulated cells following 24 h incubation. The results are expressed as the mean ± SEM from six experiments performed using cells cultured from different donor tissue. $^*P < 0·05$.

cells, actinomycin D and trifluoperazine partially reduced and tunicamycin significantly ($P < 0·05$) reduced release of PGE_2, while in the neonatal cells PGE_2 release was significantly ($P < 0·05$) reduced in cells exposed to 10 μg/ml but not 1 μg/ml actinomycin D. Tunicamycin partially reduced release of PGE_2 but trifluoperazine did not (Table 1). LPS-induced release of PGE_2 from both neonatal and adult cells was also totally blocked by indomethacin and significantly reduced by cycloheximide ($P < 0·005$) but not by actinomycin D, tunicamycin or trifluoperazine (Table 2).

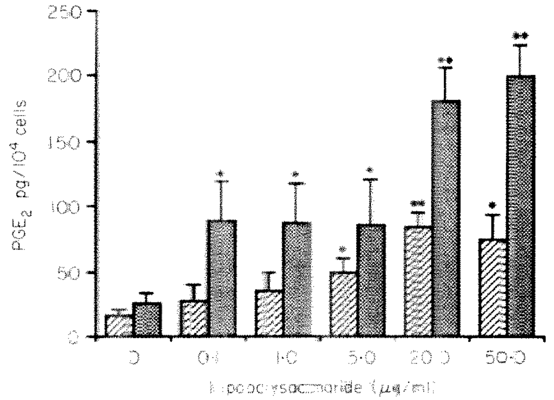

FIGURE 4. Release of PGE_2 from adult (■) and neonatal (▨) human dermal microvascular endothelial cells following 24 h incubation with concentrations of LPS between 0·1 and 50·0 μg/ml. The columns represent the mean release of PGE_2 and the bars indicate the SEM from six experiments performed using cells cultured from different donor tissue. $*P < 0{\cdot}05$, $**P < 0{\cdot}005$.

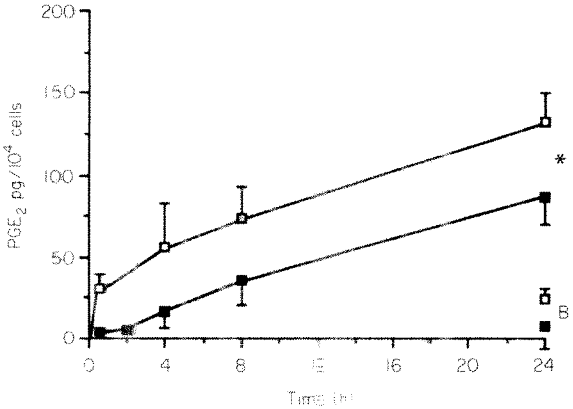

FIGURE 5. The time course of release of PGE_2 from adult (■) and neonatal (□) human dermal microvascular endothelial cells incubated with 20 μg/ml LPS. B indicates basal release of PGE_2 from unstimulated cells following 24 h incubation. The results are expressed as the mean ± SEM from six experiments performed using cells cultured from different donor tissue. $*P < 0{\cdot}05$.

DISCUSSION

Human dermal microvascular endothelial cells were successfully cultured from both neonatal foreskin and adult facial skin and the endothelial nature of the cultured cells confirmed by positive staining for vWF protein and binding of the lectin *Ulex europaeus* agglutinin-1. Of the three mAbs examined, only EN 4 gave consistent positive staining under the fixation conditions used. Positive staining with PAL-E and BMA 120 may have been observed using different fixation conditions, but neither PAL-E nor BMA 120 were raised against endothelial cells. PAL-E was raised against human lymph node metastases of melanoma[15] and BMA 120 against an established human oat-cell carcinoma cell line,[16] suggesting that these antibodies may be directed against epitopes unique to tumour vasculature or rapidly proliferating endothelium.

TABLE 1. The effect of metabolic antagonists on hrIL-1α-induced release of PGE_2 from adult dermal microvascular endothelial cells

		Adult cells	Neonatal cells
Indomethacin	10 μM	0·0**	0·71 ± 0·71**
Cycloheximide	50 ng/ml	11·23 ± 6·84**	19·09 ± 3·09**
Actinomycin D	1 ng/ml	74·17 ± 10·75	101·99 ± 10·82
	10 ng/ml	61·55 ± 10·26	31·60 ± 9·89*
Tunicamycin	1 μg/ml	45·66 ± 14·90*	60·29 ± 16·78
Trifluoperazine	100 ng/ml	57·54 ± 13·99	107·86 ± 10·91

Confluent monolayers of endothelial cells were pre-incubated with the antagonists for 30 min prior to the addition of 1 U/ml hrIL-1α. The incubations were then continued for 6 h. The results are expressed as % PGE_2 released in the presence of the antagonists compared to PGE_2 released in the presence of hrIL-1α alone and represent the mean ± SEM from four experiments performed using cells cultured from different donors *$P < 0·05$, **$P < 0·005$

TABLE 2 The effect of metabolic antagonists on LPS-induced release of PGE_2 from dermal microvascular endothelial cells

		Adult cells	Neonatal cells
Indomethacin	10 μM	4·69 ± 4·69**	4·12 ± 4·12**
Cycloheximide	50 ng/ml	21·53 ± 7·24**	17·45 ± 9·84**
Actinomycin D	1 ng/ml	174·54 ± 45·98	90·90 ± 7·63
Tunicamycin	1 μg/ml	93·48 ± 21·35	101·22 ± 25·59
Trifluoperazine	100 ng/ml	102·14 ± 11·58	101·20 ± 17·21

Confluent monolayers of endothelial cells were pre-incubated with the antagonists for 30 min prior to the addition of 20 μg/ml LPS. The incubations were then continued for 6 h. The results are expressed as % PGE_2 released in the presence of the antagonists compared to PGE_2 released in the presence of LPS alone and represent the mean ± SEM from four experiments performed using cells cultured from different donors. **$P < 0·005$

Our results show that human dermal microvascular endothelial cells cultured from both neonatal foreskin and adult facial skin release PGE_2, but not PGI_2, when incubated with either the pro-inflammatory cytokine IL-1 or Gram-negative bacterial endotoxin (lipopolysaccharide). LPS-induced release of PGE_2 was initially slow, with PGE_2 first being detectable at between 2 and 4 h after stimulation, but was then continuous up to 24 h. In contast, release of PGE_2 from human dermal microvascular endothelial cells incubated with hrIL-1α, increased most rapidly up to 6 hours and then more slowly up to 24 h.

There appeared to be a requirement for new protein synthesis for continued release of PGE_2, as release induced by both LPS and hrIL-1α was consistently and significantly inhibited by cycloheximide. Actinomycin D partially inhibited release of PGE_2 from cells incubated with hrIL-1α but did not inhibit LPS induced release. This differential effect of actinomycin D as well as tunicamycin and trifluoperazine possibly indicates different mechanisms of cell activation. The LPS-induced release of PGE_2 from adult cells exposed to actinomycin D may be due to a combined toxic effect of actinomycin D and LPS together. Metabolic antagonists are cytotoxic at high concentrations and the degree of tolerance varies between different cell types. Although the concentrations of actinomycin D used in our experiments with dermal microvascular endothelial cells were lower than used by others in fibroblasts and smooth muscle cells,[18,19] they had an effect on cells incubated with hrIL-1α without being cytotoxic, as shown by the partial inhibition of hrIL-1α release of PGE_2. Higher concentrations might well have been more potent inhibitors of PGE_2 release, but they were cytotoxic for human dermal microvascular endothelial cells in culture. The reduced efficacy of actinomycin D in the adult cells may be associated with their reduced responsiveness to the agonist compared to the neonatal cells.

There is now evidence that regulation of prostaglandin synthesis is mediated by at least two mechanisms, one involving stimulus-induced activation of pre-existing enzymes and the other mediated by protein synthesis. We have recently reported that histamine can induce release of PGI_2 from human large-vessel endothelial cells cultured from umbilical cords for up to 24 h by a mechanism that does not exhibit a requirement for protein synthesis.[20] This is in contrast to results reported here, where hrIL-1α and LPS-induced release of PGE_2 is apparently mediated by protein synthesis. Whether this is specific induction of enzyme protein is not known, but it has recently been reported that in human dermal fibroblasts, hrIL-1-induced synthesis of PGE_2 is mediated primarily, if not solely, via induction of cyclo-oxygenase synthesis in a manner that is time- and concentration-dependent.[21]

We did not detect 6keto-$PGF_{1\alpha}$ in the supernatant of either neonatal or adult human dermal microvascular endothelial cells incubated with LPS or hrIL-1α using a radioimmunoassay sensitive at the 5 pg level. These findings are in contrast to those of Charo *et al.*[8] who detected low levels of 6keto-$PGF_{1\alpha}$ in the supernatant of neonatal cells stimulated with either the calcium ionophore A23187 or thrombin. These authors also demonstrated release of $PGF_{2\alpha}$ and the cross-reactivity of their 6keto-$PGF_{1\alpha}$ antibody with $PGF_{2\alpha}$ was 2·2%. The 6keto-$PGF_{1\alpha}$ antibody used in our experiments exhibited 0·5% cross-reactivity with $PGF_{2\alpha}$ which may account for the differences observed. Reduced capacity to release PGI_2 appears to be a feature of microvascular endothelial cells, as both rabbit coronary[22] and guinea-pig brain capillary-endothelial cells[23] have also been shown to release less 6keto-$PGF_{1\alpha}$ than PGE_2, indicating that PGI_2 is not an important arachidonic acid metabolite in the microvascular endothelium.

As well as being a potent vasodilator, PGE_2 is mitogenic for both capillary endothelial cells[24] and epidermal keratinocytes.[25] It appears to regulate a variety of cellular responses to pro-inflammatory stimuli, including down regulation of T-lymphocyte responses[26] and inhibition of monocyte IL-1 synthesis by elevating intracellular cyclic AMP levels.[27] The initial rapid, and subsequently more gradual, release of PGE_2 from hrIL-1α-stimulated human dermal microvascular endothelial cells may indicate that PGE_2 is auto-regulating its own release by down-regulating the effect of the hrIL-1α.

A consistent observation in these experiments was the reduced sensitivity to the same stimulus, of microvascular endothelial cells derived from adult tissue, compared to those cultured from neonatal tissue. This may represent differences between cells cultured from skin of different anatomical sites or alternatively it may represent differences between cells cultured from aged compared with young tissue, as has recently been observed with cultured keratinocytes (B.A.Gilchrest, personal communication).

These results indicate that the dermal microvasculature is a target for pro-inflammatory mediators and that it is also a source of vasoactive mediators as shown by the protracted release of PGE_2 following exposure to both hrIL-1α and LPS. Furthermore, these data indicate an important and active role for the human dermal microvascular endothelium in cutaneous inflammatory reactions.

ACKNOWLEDGMENTS

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Pharmacodynamic measurements of methyl nicotinate percutaneous absorption: the effect of aging on microcirculation

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SUMMARY

The penetration of drugs through aged skin is important both in terms of transdermal delivery to elicit systemic pharmacological effects, and for topical treatment. Cutaneous microcirculation efficiency, an integral parameter in the overall process of percutaneous absorption, was studied in young (20–34 years) and old (64–86 years) individuals. Cutaneous erythema as induced by topical administration of methyl nicotinate to the ventral forearm, was monitored non-invasively using laser-Doppler flowmetry. Dose–response behaviour was characterized by five parameters: (i) the time of onset of action; (ii) the time to reach maximum response; (iii) the magnitude of the maximum response; (iv) the area under the response–time curve; and (v) the time to decay to 75% of the maximum response. Additionally, the sensitivity and efficiency of the cutaneous microcirculation in both age groups was evaluated using a pharmacokinetic–pharmacodynamic model. Statistical analysis of all data showed no significant differences between the age groups for the same concentrations. The results indicate that microvessel reactivity to the applied stimulus is comparable in the ventral forearm of both young and old populations.

Human skin undergoes numerous physiological and biochemical alterations with increasing age, including increased dryness of the stratum corneum, a reduction in sebaceous gland activity (and decrease in skin surface lipids), flattening of the dermo–epidermal junction, and atrophy of the dermal capillary network.[1]

Transdermally delivered chemicals, which have diffused across the epidermis into the dermis, are taken up by the cutaneous capillary network into the systemic circulation. Therefore the progressive atrophy of the dermal microcirculation with increasing age may cause a decrease

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in the absorption of penetrating substances. Previous work involving dermal clearance measurements,[2,3] and the McClure–Aldrich test,[4,5] has shown impairment, but contradictory findings have also been reported.[6] More recently, an investigation[7] into the effect of aging on microvessel reactivity following topical administration of the local vasodilator, methyl nicotinate (MN), showed no significant differences in the response to the stimulus between the young and old populations studied. However, the procedure and data analysis did not differentiate between possible changes in either microcirculation sensitivity or barrier function. The methodology recorded the summation of the two processes, and did not consider the possibility of compensatory alterations in the efficiency of the two events.

This study investigated whether the potent vasodilator, MN, could be used to evaluate the efficiency of the microcirculation as a function of increasing age *in vivo*. The time-course of the local effects of MN has been assessed by laser-Doppler flowmetry (LDF). MN penetrates the epidermis and acts upon the dermal capillaries. Because only finite doses of MN are applied, the vasoresponse passes through a maximum and then decays, consistent with uptake of agent into the capillaries and systemic dilution. We evaluated the kinetic processes of local skin absorption, cutaneous reactivity and dermal elimination of MN and analysed the results using a pharmacokinetic–pharmacodynamic model.[8]

METHODS

Laser-Doppler flowmetry

The pharmacological response to topically administered MN was monitored with a laser Doppler flowmeter (Periflux Pf2, Perimed KB, Stockholm, Sweden) interfaced to a flatbed pen recorder (YEW Yokogawa Hokushin Electric Corporation, Tokyo, Japan). The LDF technique has been described in detail elsewhere.[9]

Experimental

Dose–response behaviour following topical application of MN was measured. Aqueous solutions of methyl nicotinate (Sigma Chemical Co., St. Louis, MO, U.S.A.) at concentrations of 2·5, 5·0, 10·0 and 25·0 mM were used; the pH of these solutions was 5·3 ± 0·9.

MN solution was applied via a saturated patch (1·0 cm diameter) (Al-test, Imeco ab, Sodertalje, Sweden), which held 50 μl (± 2%) of solution. Application time was 15 s, after which the patch was removed and the site carefully dried with absorbent tissue. The LDF probe was then immediately positioned at the centre of the application site and blood-flow response was continuously monitored for the next 90 min. All response measurements were normalized, i.e. corrected for pretreatment baseline flow and chart recorder drift (< 30 mV).

The experimental subjects were 13 (seven male, six female) Caucasian adults, having no history of dermatologic disease, divided into two groups: young (20–34 years; $n = 6$) and old (64–86 years; $n = 7$). The subjects were allowed no prescription nor over-the-counter medication for the duration of the study. Subjects were non-smokers and refrained from caffeine and alcohol for 24 h prior to each test. Successive measurements in each individual were performed at least 3 days apart.

The experiments were conducted in a well-ventilated room under stable temperature and humidity conditions ($T = 23° \pm 2°C$; relative humidity = 30–50%). The subjects rested in a supine position throughout the measurement and were kept in the test environment 20–30 min prior to each experiment. The test site was the ventral surface of the forearm, 9 cm below the elbow fold. A control site, 5 cm below the elbow fold, was monitored for spontaneous changes in baseline perfusion. Each concentration was measured separately on both forearms. Baseline measurements were taken at both sites until a stable reading was obtained; typically, the latter was achieved within 5–7 min.

RESULTS

LDF response curves as a function of time were similar to those previously reported.[8] Five parameters characterized the time course and extent of the normalized pharmacodynamic response: (i) the magnitude of the maximum response (mV) (Fig. 1); (ii) the area under the response–time curve (mV h) (Fig. 2); (iii) the time of onset of action (min), defined as the greatest change in response per unit time (Fig. 3); (iv) the time (min) of maximum response (Fig. 4); (v) the time (min) required for the response to return to 75% of the maximum achieved (Fig. 5). Statistical comparisons of these parameters involved a one-way analysis of variance, followed by

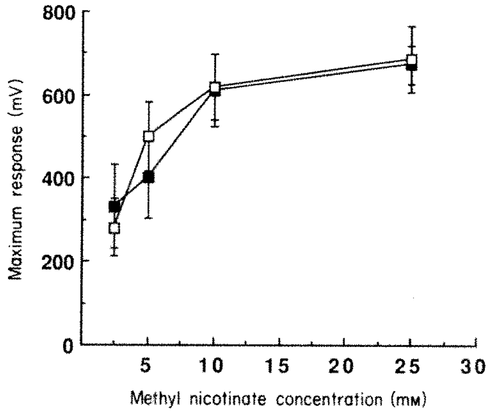

FIGURE 1. Normalized maximum response (mean ± SE) as a function of applied MN concentration: young (■) and old (□).

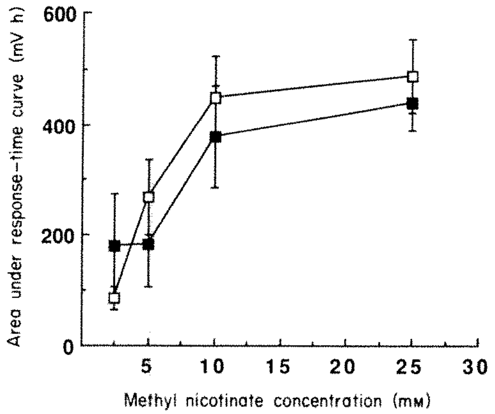

FIGURE 2. Normalized area under the response–time curve (mean ± SE) as a function of applied MN concentration: young (■) and old (□).

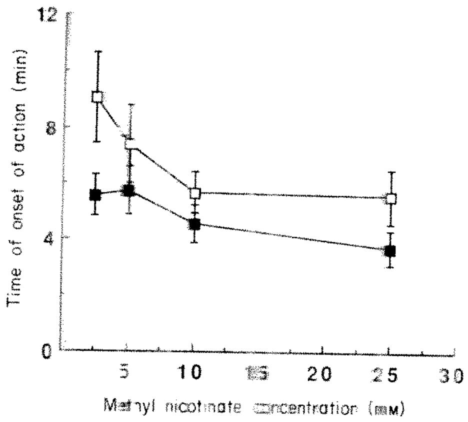

FIGURE 3. Time of onset of action (mean ± SE) as a function of applied MN concentration: young (■) and old (□).

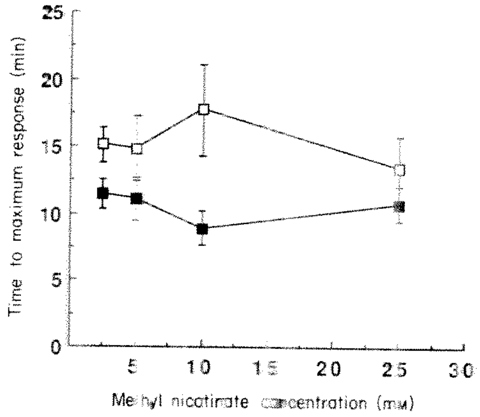

FIGURE 4. Time of maximum response (mean ± SE) as a function of applied MN concentration: young (■) and old (□).

a Newman–Keuls multiple-range test. Significant differences were found only when the responses to different MN concentrations were compared (i.e., the response to 2·5 mM MN in the old subjects was less than that to both 10 mM and 25 mM MN in the same group). At the same MN concentration applied, no significant differences were found between the young and old groups at the $\alpha = 0{\cdot}05$ level.

A trend toward saturability of the local microvascular response was apparent in the plots of normalized maximum response, and area under the response–time curve, versus applied concentration (Figs 1 and 2, respectively). These figures show (and confirm[8]) that dose–response behaviour was investigated using concentrations below saturation of the pharmacodynamic response, i.e. in the linear portion of the effect versus applied concentration curve. The onset of action (Fig. 3) and the time of maximum response (Fig. 4) are both weakly dose-dependent, i.e. the higher the concentration applied, the shorter the time of onset and the time to peak response. The time to decay to 75% of the peak response (Fig. 5) is also dose-dependent,

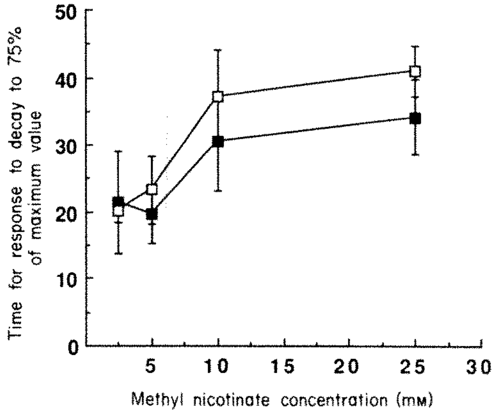

FIGURE 5. Time to decrease to 75% of the peak response (mean ± SE) as a function of applied MN concentration: young (■) and old (□).

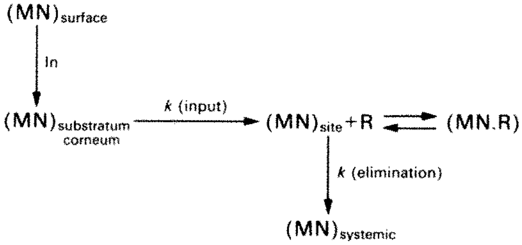

FIGURE 6. Pharmacokinetic–pharmacodynamic model (adapted from reference 8).

i.e. the higher the concentration, the longer the time required for the vasodilation to decrease to 75% of the maximum response. These results agree with those previously published.[7]

The dose–response behaviour associated with topical MN application was further analysed using a pharmacokinetic–pharmacodynamic model[8] (Fig. 6) which assumes that: (i) delivery of MN across the stratum corneum is constant during application of the patch; (ii) following input of MN to the skin, drug diffuses to its site of action with first-order kinetics (k_i);[10] (iii) loss of MN from the site of action (via capillary uptake) is a first-order elimination function (k_e).[11] The model leads to an expression, based on the Hill equation, relating applied MN concentration to the observed pharmacologic effect:[8]

$$(E - E_o) = \frac{E_{max}\, R^N}{R^N + C_{ss,50}} \tag{1}$$

where

$$R = k(C_{app})(t_{app})u \tag{2}$$

and

$$u = k(t - t_{lag}) \exp[-k(t - t_{lag})]. \tag{3}$$

E is the measured response, E_o is the baseline perfusion level, E_{max} is the maximum response, $C_{ss,50}$ is the hypothetical patch concentration of nicotinate, which would be necessary to maintain, under steady-state delivery conditions $(E - E_o) = 0{\cdot}5\, E_{max}$ (assuming no tolerance), N is a parameter affecting the steepness of the effect versus dose curve, C_{app} is the applied MN

TABLE 1. Pharmacodynamic model parameters (mean ± SE)*

Group	k (min^{-1})	$C_{ss,50}$ (mM)	E_{max} (mV)	N
Young ($n=6$)	0·109 ± 0·013	0·09 ± 0·02	756 ± 24	2·60 ± 0·32
Old ($n=7$)	0·090 ± 0·011	0·07 ± 0·02	743 ± 39	3·15 ± 0·23

* Based on a repeated measures ANOVA, there was no significant difference ($P > 0·05$) between the parameter values characterizing the young group and those describing the old cohort. Further analysis of k and $C_{ss,50}$ values, involving logarithmic transformation of the data to reduce outliers, also failed to reveal any statistically significant differences between young and old populations

concentration in the patch, and t_{app} is the patch application time (0·25 min). The concentration of drug at the site of action is controlled by the input (k_i) and elimination (k_e) kinetics; however, the results do not allow k_i and k_e to be differentiated because their values are similar.[8] Consequently, $k = k_i = k_e$ are set and a single exponential expression for u (the normalized concentration of MN within the skin) results (eqn 3). This equation incorporates a lag time (t_{lag}), which is the observed period between drug application and response onset.

The experimental data were fitted to equation (1) using MKMODEL, a parameter-estimation modelling procedure which uses a least-squares regression program.[12] Parameter values for k, $C_{ss,50}$, E_{max} and N were identified for each set of results. To reduce the number of parameters being fitted at any one time, C_{app}, E_o and t_{lag} were fixed: C_{app} for obvious reasons, E_o and t_{lag} because they can be directly obtained from the data. Average parameter estimates for both 'populations' were found and compared (Table 1). No significant differences ($P < 0·05$) in the parameters characterizing the two age groups were found.

DISCUSSION

The results shown in Figures 1–5 indicate a clear relationship between applied MN concentration and blood-flow response. However, the data indicate no significant difference in the microcirculatory response to MN between the two age groups. The pharmacodynamic changes observed require penetration to, and stimulation of, the microvasculature, and the parameters characterizing the response were chosen to highlight possible differences in these processes between the subject groups. For example, if more chemical is absorbed during the 15 s exposure period, the extent of vasoresponse (i.e. the area under the response–time curve) should be enhanced. If the penetration barrier is lower, a faster onset of response may be expected; and, if the ability of the microcirculation to eliminate the penetrated drug is compromised, the time for recovery of blood flow to pre-application levels should be increased.

Parameter estimation from the pharmacodynamic model analysis indicates that the half-lives (mean ± SE) for MN delivery to, and elimination from, the site of action are: 6·4 ± 0·8 min for the young subjects; 7·7 ± 1·0 min for the old group. The difference is not significant; nevertheless, the values agree with previous non-invasive measurements using this vasodilator.[13,14] They are also consistent with the results of a different experimental approach,[15] which showed that the life span of MN in the dermis was 3–10 min. It should be noted that the clearance half-life of Na^{22}Cl has been determined to be of the same order.[2,3,6] However, although the techniques used in these various investigations are different, either the chemical studied (MN[13-15]) or the method of delivery (intradermal injection[2,3,6]) causes local vasodilatation. Agreement between k_e values should, therefore, be used only for comparative purposes. The $C_{ss,50}$ values, which measure the sensitivity of the microcirculation to drug-induced vasodilatation, showed no significant difference between age groups.

Evaluation of the results and further pharmacodynamic analysis showed no significant differences between age groups. This may be due in part to the high inter-individual variability observed (coefficients of variation of the model parameters ranged from 22 to > 94%). Although strict experimental procedures were followed, this degree of variability appears to be characteristic for this type of measurement.[7,8] However, the results agree with current observations concerning the effect of skin aging on percutaneous penetration. If there is no difference in dermal capillary uptake of penetrating substances in the forearm area, but barrier function increases with age, then the important factor is the role of the stratum corneum which is the major barrier to diffusion of penetrating substances. Subtle changes in the stratum corneum barrier (e.g., in intercellular lipid organization) may account for changes seen in penetration, independent of microcirculatory uptake.

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The effect of cyclosporin on human epidermal keratinocytes *in vitro*

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SUMMARY

Cyclosporin A has been shown to be effective in the treatment of severe, recalcitrant psoriasis, but it is uncertain whether the mode of action is primarily by immune suppression or by other mechanisms. Cyclosporin-dependent growth-inhibition has recently been demonstrated *in vitro* using several non-human and transformed epithelial cell lines. In this study the effect of cyclosporin on human epidermal keratinocytes and skin fibroblasts was investigated. Secondary cultures of human epidermal keratinocytes were grown on collagen-coated dishes in the presence of increasing concentrations of cyclosporin. Inhibition of growth was observed at 6–8 μM. An almost identical dose–response curve was obtained for the cytotoxic drug, *cis*-platin. Short-term exposure (1 h) to cyclosporin did not have any effect on epidermal cell growth, suggesting that direct membrane-related effects were not involved. Analysis of cellular proteins by SDS–PAGE indicated no effect of continuous cyclosporin exposure on *in vitro* differentiation. The observation that human epidermal keratinocyte growth is inhibited by cyclosporin suggests that a topical form of therapy for psoriasis may be an effective alternative to oral treatment.

Cyclosporin A is a cyclic polypeptide of 11 amino acids with potent immunosuppressive properties. It has been used both experimentally in animals and clinically in man to prolong allogenic transplants. More recently it has been used at lower doses to treat severe psoriasis[1–3] with rapid clearing of the lesions, even in previously recalcitrant cases. At present it is not certain whether these effects are primarily due to an alteration of immune function, particularly T-cell modulation, or to a direct effect on the skin.

This study examined the effect of cyclosporin on human epidermal keratinocytes and human skin fibroblasts *in vitro*. As well as providing information as to the possible mode of action of

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cyclosporin *in vivo*, such an approach may help decide whether topical cyclosporin is a possible alternative treatment in psoriasis, thus minimizing the known side-effects of oral cyclosporin such as nephrotoxicity.

METHODS

Fibroblast cultures

Human fibroblast cultures were derived from normal adult human trunk skin removed during routine minor surgical procedures. Cultures were established by routine procedures and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum.

Epidermal keratinocyte cultures

Human epidermal keratinocyte cell suspensions were prepared from foreskins and plated out onto mitomycin-treated 3T3 fibroblasts as previously described.[4] Keratinocytes were maintained in a mixture of DMEM:Ham's F12 (3:1) containing 5% fetal calf serum, 0·4 μg/ml hydrocortisone, 5 μg/ml insulin, 10 ng/ml cholera toxin, 5 μg/ml transferrin, 10 ng/ml human epidermal growth factor (a gift from Dr H.Gregory, ICI Pharmaceuticals Ltd, Macclesfield, U.K.) and 2×10^{-11} M triiodo-thyronine. During the first 7 days of primary culture the medium also contained penicillin (100 U/ml) and streptomycin (100 μg/ml).

Growth inhibition studies

Primary cultures of human epidermal keratinocytes were trypsinized and resuspended and 1·0–2·0 × 10^5 cells plated out onto rat-tail-tendon collagen-coated 35 mm Petri dishes without 3T3 fibroblasts. The growth medium contained 5% fetal calf serum and the growth factors as described above. After 24-h culture the medium was changed to medium containing cyclosporin (Cyclosporin A, gift from Sandoz Pharmaceuticals) or fresh medium containing vehicle alone. The maximum concentration of cyclosporin was limited by its solubility to 10 μM. The cyclosporin was made up as a stock solution in ethanol:0·1% DMSO in water (50:50). The maximum concentration of vehicle did not exceed 0·5%. After a further 6 days of culture the medium was removed and the cultures washed with Dulbecco's salt solution (DSS), formalin-fixed and stained with 0·1% crystal violet. The percentage of the dish surface covered with keratinocytes was then determined using the Optomax V image analysis system. The percentage area is considered to be the best overall index of cell growth in epidermal keratinocyte cultures.[5]

Cultures of human fibroblasts between passage numbers 4 and 8 were subcultured into 35-mm Petri dishes (1·0–2·0 × 10^4 cells per dish) containing cover slips. In some experiments the concentration of newborn calf serum in the medium was reduced from 10 to 5%. After 24 h the medium was changed to medium containing cyclosporin or vehicle alone. After a further 4–6 days of culture the medium was removed and the cells washed with DSS, formalin-fixed and stained with the nuclear stain, gallocyanin. The mean number of cell nuclei per random field was determined microscopically as previously described.[4] At least 15 fields per slide were counted.

In some experiments comparisons were made with *cis*-platin and methotrexate in terms of their growth inhibition at similar concentrations to cyclosporin. Stock solutions of *cis*-platin were made up in DSS, whereas stock solutions of methotrexate were made up in 0·01 M NaOH.

Cell survival experiments

Primary cultures of epidermal keratinocytes were incubated with either 10 μM cyclosporin or *cis*-platin for 1 h. After washing, the cultures were subcultured onto collagen and grown without 3T3 fibroblasts. The growth was assessed after 6 days as described above.

SDS-PAGE electrophoresis

Cultures of human epidermal keratinocytes treated with either 10 μM cyclosporin or vehicle alone for a period of 6 days were extracted with 20 mM Tris-HCl, pH 7·4 and then re-extracted with 20 mM Tris-HCl pH 7·4 containing 8 M urea and 1% β-mercaptoethanol (keratin-containing fraction). Both extracts were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described.[6]

RESULTS

The results of two experiments to determine the effect of cyclosporin on the growth of human epidermal keratinocytes are given in Figure 1. In both cases there is a clear dose-response curve showing inhibition of keratinocyte growth with increasing concentrations of cyclosporin. Keratinocytes grown in the presence of cyclosporin showed a flattened, irregular appearance suggesting that the pattern of *in vitro* differentiation may have been altered. However the results of the SDS-PAGE (Fig. 2) did not confirm this. No major differences in the polypeptide pattern were seen between the control and 10 μM cyclosporin-treated cells in the Tris-HCl extract and the keratin-containing fraction. Analysis from a second separate experiment also failed to demonstrate significant differences between the polypeptide patterns. Similarly, electron microscopic examination (results not shown) did not reveal any ultrastructural differences between control- and cyclosporin-treated cultures.

The results of experiments with *cis*-platin (Fig 3a) indicated that the growth-inhibition of keratinocytes observed with cyclosporin was at similar levels to that observed with *cis*-platin. Thus, a value of approximately 50% of control value was reached at 8-10 μM for cyclosporin compared to 10 μM for *cis*-platin. Comparisons with methotrexate were not possible in this situation as the dose response curve was very flat despite the wide range (0·1 μM-1 mM) of concentrations used (Fig. 3b).

The results of two experiments to determine the effect of cyclosporin on human fibroblasts are given in Table 1. Although there appeared to be a slight decrease in the numbers of cells with

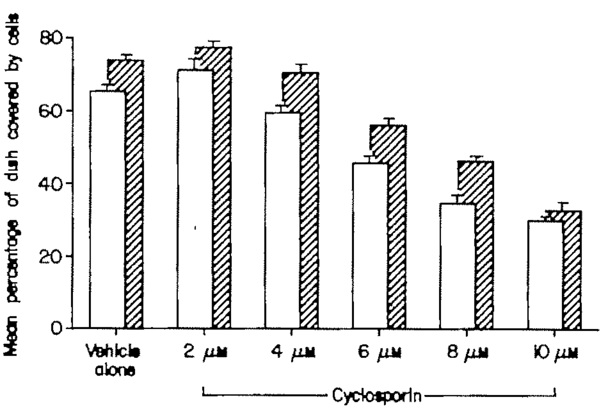

FIGURE 1. The effect of cyclosporin A on the growth of human epidermal keratinocytes. Results are expressed as the mean ± SEM of three dishes. ■, experiment 1; □, experiment 2.

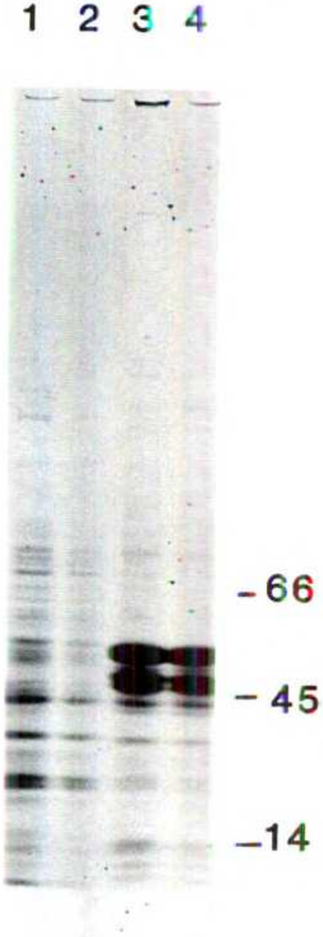

FIGURE 2. SDS–PAGE electrophoresis of extracts of epidermal keratinocytes. Tris/HCl extract of vehicle treated (1) or 10 μM cyclosporin treated (2) keratinocytes. Keratin fraction of vehicle treated (3) or 10 μM cyclosporin treated (4) keratinocytes. Molecular weight markers used were bovine serum albumin (66 K), egg albumen (45 K) and lysozyme (14 K).

increasing concentration of cyclosporin in medium containing 10% serum the effect was only minimal and much less marked than with keratinocytes. In contrast, when the experiment was repeated in the presence of 5% serum a marked effect was noted. There were some changes in the morphology of fibroblasts when grown in cyclosporin, the cells being less spread out and with a denser cytoplasm. The effect of *cis*-platin on fibroblasts in the presence of 10% serum is shown in Figure 4a. A notable decrease of cell numbers was observed with an IC_{50} value below the range of concentrations tested. A similar response was observed with methotrexate (Fig. 4b). No difference was noted with these two drugs on reduction of the serum concentration to 5%.

The results of two separate cell-survival experiments are given in Table 2. The short-term

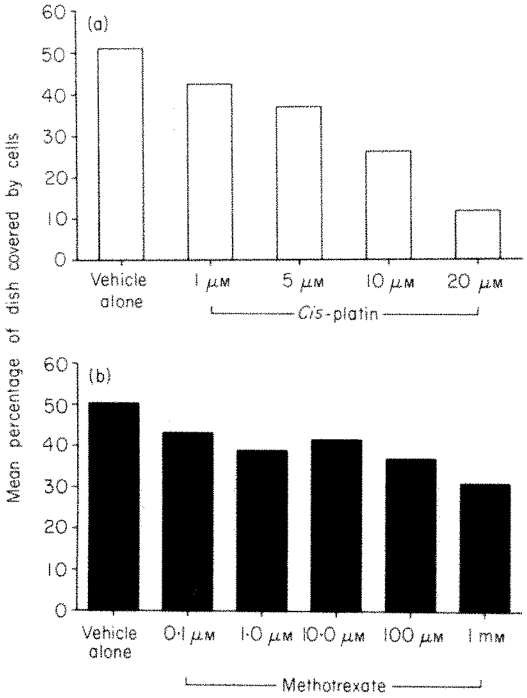

FIGURE 3. The effect of (a) *cis*-platin and (b) methotrexate on the growth of human epidermal keratinocytes. Results are expressed as the mean (a, $n=3$, b, $n=2$).

TABLE 1. The effect of serum on the sensitivity of human dermal fibroblasts to cyclosporin

	Cyclosporin concentration				
	2 μM	4 μM	6 μM	8 μM	10 μM
10% serum (%)	96·56*	92·14	97·75	92·12	90·10
5% serum (%)	81·16	81·20	72·73	43·37	25·19

*Results are expressed as the percentage of the mean number of nuclei in control dishes receiving vehicle alone. A minimum of three dishes were used at each concentration.

exposure of keratinocytes to cyclosporin or *cis*-platin did not result in any change in the cellular growth rate.

DISCUSSION

The results of early studies on the effects of cyclosporin on epidermal cells indicated that there was little or no direct effect *in vitro*. Cyclosporin did not inhibit the outgrowth of pig epidermal explants at therapeutic levels (200 ng/ml = 0·17 μM).[7] No effect on human epidermal keratinocytes at concentrations up to 10 μM was reported.[8] However, inhibition of normal

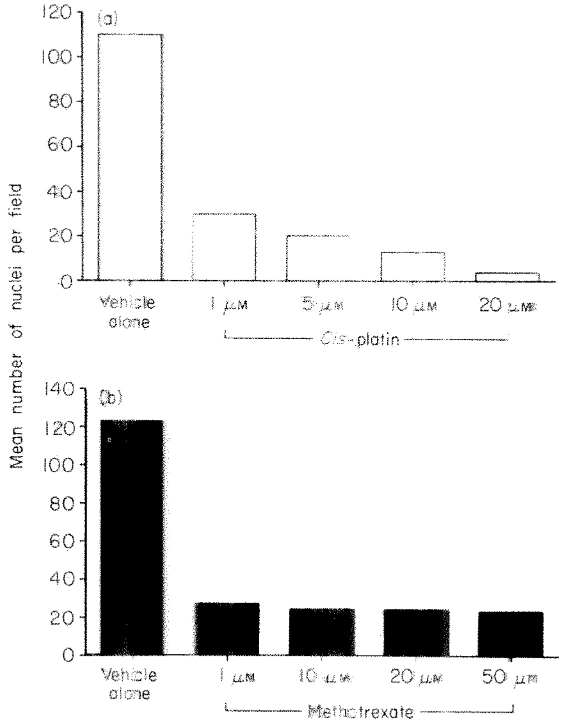

FIGURE 4. The effect of (a) *cis*-platin and (b) methotrexate on the growth of human fibroblasts. Results are expressed as the mean two dishes.

TABLE 2. The effect of short term exposure to cyclosporin or *cis*-platin on human epidermal keratinocytes

	Experiment 1			Experiment 2		
	Dish	% Area	Mean ± SEM	Dish	% Area	Mean ± SEM
Control	1	40·16		1	58·34	
	2	47·08	44·59 ± 2·23	2	60·54	57·22
	3	46·54		3	52·79	± 2·31
Cyclosporin (10 μM)	1	47·02		1	51·28	
	2	48·93	45·72 ± 2·32	2	55·97	53·24
	3	41·22		3	53·38	± 1·10
Cis-platin (10 μM)	1	43·33		1	57·12	
	2	42·98	44·35 ± 1·21	2	59·35	56·01
	3	46·78		3	51·56	± 2·32

mouse keratinocytes was demonstrated, as well as with transformed mouse keratinocytes and A431 cells, at concentrations ranging from 1 to 10 μg/ml (0·83–8·3 μM).[9] More recent studies have shown some inhibitory effects of cyclosporin on human epidermal cells at similar levels but only in serum free medium.[10]

The results of this study show that cyclosporin inhibits the growth of human epidermal keratinocytes in serum-containing medium, when exposure is continuous over a period of several days. Previous studies[10] were not able to demonstrate effects in the presence of serum. This may have been due to the shorter exposure to cyclosporin (48 h) or the inherent unreliability of using [^{3}H]-thymidine-uptake as a measure of epidermal cell growth.[11,12] Other workers have found cyclosporin to inhibit growth in long-term culture in the presence of serum using A431 cells.[9] As demonstrated with fibroblasts, the concentration of serum can affect the dose–response curve for cyclosporin. This, together with the inevitable inter-laboratory variation in culture techniques and experimental design, probably explains the failure of some attempts to demonstrate cyclosporin effects *in vitro*.

The inhibition of epidermal keratinocyte growth by cyclosporin seen in this study was comparable to that noted with the cytotoxic drug, *cis*-platin. The lack of effect of methotrexate may be due to the active thymidine-salvage pathway present in keratinocytes.[13] The levels of cyclosporin needed to produce the effect *in vitro* were comparable to those measured in skin samples taken from patients receiving systemic treatment, 1–3 μg/ml (0·83–2·49 μM).[10] This suggests that cyclosporin may act *in vivo* in patients with psoriasis partly by a direct effect on the epidermis.

The mechanism of the observed growth inhibition of human epidermal keratinocytes by cylcosporin is not clear. It has recently been reported that severe intra-uterine growth retardation occurs during treatment with cyclosporin.[14] It may be that cyclosporin has growth inhibiting properties as well as its immunoregulatory effects. It has been reported that direct inhibitory effects on keratinocytes can be demonstrated in human skin grafted onto athymic mice.[15] Alternatively cyclosporin may have some effect on differentiation, and an isolated report has indicated that cyclosporin may be effective in ichthyosis.[16] Although in our study some morphological changes were observed by phase-contrast light-microscopy, no significant ultrastructural abnormalities could be seen in the cyclosporin-treated keratinocytes. No effect on the *in vitro* differentiation, as judged by the SDS–PAGE polypeptide pattern, could be seen.

In contrast to the above findings of growth inhibition with continuous exposure to cyclosporin, short-term exposure did not effect the growth rate. This suggests that the action of cyclosporin on keratinocytes is more likely to be an intracellular and perhaps a multi-stage process, possibly by interaction with a cytosolic binding protein such as cyclophilin,[17] rather than by some direct membrane effect such as can be demonstrated within minutes in lymphocytes.[18] An alternative interpretation is that there is a direct effect on the membrane but that this is rapidly reversed upon exposure to cyclosporin-free medium.

The finding of growth inhibition of human epidermal keratinocytes *in vitro* at levels comparable to those found *in vivo* after systemic therapy,[10] makes it seem likely that a topical preparation of cyclosporin would be clinically useful in psoriasis. However, preliminary studies of topical cyclosporin in psoriasis have been disappointing,[19,20], although intralesional treatment has been reported as successful.[21] Topical cyclosporin has been shown to be effective in the suppression of allergic type dermatitis[22] and it may be that the vehicle chosen in the psoriasis studies was not the optimum for a highly lipophilic molecule such as cyclosporin. Further studies are needed to determine the rates of release and skin levels after topical application before any final conclusion as to the usefulness of topical cyclosporin can be drawn.

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The eosinophil granulocyte in psoriasis

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SUMMARY

The presence of eosinophils and eosinophil cationic protein (ECP) in the involved and non-involved skin in patients with psoriasis was studied using a polyclonal antibody specific for ECP and a monoclonal antibody (EG2) specific for activated eosinophils and secreted ECP. ECP immunoreactive eosinophils were found in all the specimens from involved psoriatic skin. In new lesions in patients with rapidly progressive disease, intense ECP immunoreactivity was detected both intra- and extracellularly, particularly in the upper third of the epidermis and usually in association with granulocytes. In stable or slowly progressive lesions, less ECP was observed. The EG2-immunoreactivity was positive in the same areas. ECP was also determined in suction-blister fluid from lesional and non-involved skin in psoriasis patients and in healthy subjects. The ECP concentration was greatly elevated in the fluid from lesions in patients with more acute and progressive disease.

Neutrophil granulocytes, monocytes and lymphocytes have been implicated in the pathogenesis of psoriasis. The role of eosinophils in psoriasis is unknown and these cells are rarely seen in routine stained sections of lesions of psoriasis.[1] A slight increase in the number of circulating eosinophils has been reported in patients with chronic, widespread psoriasis, particularly those with arthritis.[2,3]

The eosinophil is a potent pro-inflammatory cell able to phagocytize and migrate with cytotoxic[4] and neurotoxic effects.[5] The granules of this cell contain a number of proteins: eosinophil cationic protein (ECP), eosinophil peroxidase (EPO), eosinophil protein X (EPX, which is equivalent to eosinophil-derived neurotoxin, EDN) and major basic protein (MBP).[4] Increased amounts of ECP or MBP in the circulation or in the tissues have been shown to reflect an increased turnover and/or increased tissue activity of the eosinophils.[6] The major part of the mature pool of eosinophils is found in the tissues and less than 5% is present in the circulation.[7]

Difficulty in identifying activated eosinophils with routine stains has been encountered[8,9] but detection has been facilitated by the development of antibodies to ECP, EPO, EPX and MBP. With monoclonal antibodies directed against ECP/EPX in activated eosinophils but not the

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stored forms of ECP, it is now also possible to discriminate between non-activated and activated eosinophils.[9]

The initial aim of this study was to determine whether the ECP levels in the serum could give any indication of involvement of eosinophils in psoriasis and pustulosis palmoplantaris (PPP). Having found increased levels of ECP in the serum of such patients, we investigated whether ECP could also be detected locally in psoriatic skin.

METHODS

Subjects for measurements of ECP and total number of circulating eosinophils

Psoriasis patients. This group comprised 35 patients (20 female, 15 male; age range 16–65 years, mean 35 years) mainly with nummular and plaque psoriasis. None of the patients had guttate lesions. At the time of blood sampling 12 patients had less than 5% involvement of the skin surface. In two the skin was almost erythrodermic and the remaining patients had 10–40% involvement of the skin with psoriasis. Two patients with moderate psoriasis had a past history of mild arthritis but this was quiescent. None of the patients were on any systemic therapy and no topical steroids had been used in the 2 weeks prior to the study.

Pustulosis palmoplantaris patients. This group consisted of 13 females and three males, aged 24–72 years (mean 47 years). The diagnosis was based on the typical clinical picture with recurrent yellowish-brown sterile pustules on the palms and soles. None of the patients had any lesions elsewhere. Four patients were considered to have mild PPP, nine to have moderate and three to have severe PPP. None of them were on any systemic treatment at the time of blood sampling.

Control group. This group consisted of 19 healthy subjects (12 male, 7 female; 25–41 years old, mean 31 years) without any infection and who were receiving no medical treatment. They had no past history of allergic asthma, rhinitis, conjunctivitis or atopic dermatitis.

Subjects for studies of ECP and activated eosinophils in skin biopsies

Skin biopsies were performed on 11 psoriasis patients without any joint symptoms (Table 1). Four patients had active progressive nummular or plaque psoriasis with an increasing number of lesions during the last weeks. One patient (No. 14) had labile erythrodermic psoriasis with some pustulation. Five patients had a more slowly progressive type of psoriasis and one patient had thick, scaly stable plaques.

In three patients (Nos 6, 13 and 14) two to three biopsy specimens were taken. In patient No. 6 the two lesions were of the same appearance and probably of about the same age (a, b). In patient No. 14, one biopsy specimen was from an area with pustules (a) and two were from areas with erythematous papules (b and c). In patient No. 13, one specimen was from a small new lesion (a) and one from an older plaque (b).

For comparison, skin biopsy specimens were taken from two healthy women aged 20 and 47 years.

Subjects for determination of ECP in suction blister fluid

Suction blisters were raised on involved and/or non-involved skin in psoriasis patients Nos 3–12 (see Table 1). For comparison, suction blisters were raised in six healthy subjects aged 27–47 years (mean 31·7 years) on abdominal skin. Suction blisters were produced according to the

TABLE 1. Data on the psoriatic patients from whom the biopsy specimens (patients Nos 1, 2, 4–7, 10–14) and blister fluid (patient Nos 3–12) were collected

Patient No.	Age	Sex	Psoriasis involved skin (%)	ECP in blister fluid Involved skin (µg/l)	 Uninvolved skin µg/l)	ECP in serum (µg/l)
1	62	M	20 sp	—	—	—
2	65	F	5 sp	—	—	—
3	31	F	10 sp	4·3	—	46·0
4	42	F	5–10 sp	10·8	3·0	2·1
5	30	M	20–25 s	—	3·4	7·1
6	22	F	30–40 sp	13·9	4·4	30·0
7	20	M	10–15 sp	14·1	2·6	16·6
8	27	M	10 a	22·0	—	—
9	43	M	10 a	30·0	—	—
10	61	M	20–25 a	39·0	2·9	5·0
11	35	M	40 a	35·0	9·0	56·0
12	60	F	30 a	—	4·0	110·0
13	47	M	30–40 a	—	—	50·0
14	57	M	>75 e	—	—	—

sp = slow progression, psoriatic lesions during the last month.
s = stable psoriasis.
a = more active progression, psoriatic lesions during the last weeks.
e = erythrodermic psoriasis with pustules.

method of Kiistala[10] with a suction power of 200–300 mmHg and the fluid was collected after 1–3 h and stored at −20°C.

ECP assay in serum and blister fluid

Blood samples were taken and serum separated off 1 h after complete coagulation. The serum was frozen and stored at −20°C.

ECP was measured by a radioimmunoassay as described by Venge.[11] Standard solution or sample (0·1 ml) was incubated with 0·5 ml Sepharex-bound anti-rabbit ECP for 2 h before addition of 0·1 ml ^{125}I ECP (40 000 c.p.m.). The incubation was then continued for 20 h at 22°C during rotation. Thereafter the sample was washed four times in 0·9% NaCl containing 0·2% cetyl methylammonium bromide (CTAB). The radioactivity of the pellet was determined in a scintillation counter.

The blister fluid was diluted in assay buffer 1:4 and the ECP concentration was measured with the same method as ECP in serum.

The sensitivity of the assay was 1 µg/l and the analytical error (coefficient of variation %, CV%) was 6–9%.

Total eosinophil count in the blood.

The total number of eosinophils in the blood was determined with a Technicon H1 analyser (Technicon Instruments Co, Tanytown, NY, U.S.A.).

Skin biopsy specimens

The specimens (3 mm) were fixed for 4 h in fresh 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) at pH 7·4, and embedded in paraffin. Serial 6-μm thick sections were treated with immunoperoxidase, immunoalkaline phosphatase or haematoxylin-eosin. Two antibodies, polyclonal ECP (directed against storage and secreted forms of ECP) and monoclonal EG2 (directed against ECP and EPX in activated eosinophils and secreted proteins) were used. The production and purification of ECP and EG2 antibodies were as previously described.[5,9]

The immunoperoxidase method and peroxidase–anti-peroxidase (PAP) procedure

The PAP procedure was used for detection of the ECP antibodies. For the immunoperoxidase method the endogenous peroxidase activity was blocked by 50 ml of 3% hydrogen peroxide in 200 ml of absolute methanol for 20 min. The sections were then gently rinsed in buffer. After soaking for 30 min in Tris-buffered saline (TBS), pH 7·4, containing 1% Triton X-100 (Sigma Chemical Co, St Louis, MO, U.S.A., Triton–TBS), sections were exposed to a 1% solution of swine serum for 30 min, to reduce non-specific background-staining, rinsed briefly in Triton-TBS and exposed to optimally diluted antiserum (polyclonal rabbit ECP antibody diluted 1:2000) for 20 h at 4°C followed by 2 h of re-equilibration at room temperature. The site of the antigen–antibody reaction was demonstrated by the PAP procedure of Sternberger.[12] Some sections were counterstained with Harris haematoxylin.

Conventional positive and negative staining controls as detailed by Sternberger,[12] absorptional (specificity) controls (40 μg ECP in 1 ml diluted antibody) and replacement of primary antibody by non-immune rabbit serum were carried out.

The APAAP procedure (alkaline phosphatase monoclonal anti-alkaline phosphatase complex)

The APAAP procedure was used for detection of the EG2 antibodies. Sections were incubated for 30 min at room temperature with the primary mouse monoclonal antibody EG2 (TriChem, DK-2830 Virum, Denmark). After a short rinse (1% Triton–TBS), anti-mouse immunoglobulin (Dakopatts dilution 1:25) was added, and the sections were incubated for 30 min at room temperature. The sections were then washed in 1% Triton–TBS for 2 min and incubated with the APAAP complex (Dakopatts, Dako Corporation, Santa Barbara, CA, U.S.A.) at a dilution of 1:50 in a moist chamber at room temperature. On incubation with an alkaline phosphatase substrate (Naphthol AS-MX phosphate and fast red TR salt with levamisole to inhibit endogenous alkaline phosphatase), a red colour developed. Conventional positive and negative staining controls and replacement of the primary antibody (EG2) by mouse monoclonal antibody of the same subclass (IgG1) were used as controls. Observations of a strong correlation between the two antibodies (the polyclonal ECP antibody and EG2) have recently been published.[13]

Statistics

For circulating eosinophils and ECP in serum, analysis of variance was used and based on logarithmically transformed data. The sex of the patients and subjects and the group affiliation, and any correlations between these were taken into account in the analyses.

For ECP in suction-blister fluid the following comparisons were made: (a) control skin vs. non-involved psoriatic skin; (b) control skin vs. psoriatic lesions; (c) psoriatic lesions vs. non-involved psoriatic skin and (d) lesions in active progressive psoriasis vs. lesions in slowly progressive or stable psoriasis. For each comparison a test of normality was performed.[14] If the hypothesis of normality could not be rejected, the two-sample *t*-test (a, d) or the matched-pair *t*-test (c) was used, depending on the data structure. If normality could not be assumed, the Mann–Whitney *U*-test (b) was applied.

RESULTS

ECP in serum

There were no significant differences in the geometric mean of ECP in serum between men and women.

An almost significant difference between the groups was found when sex was taken into account ($P = 0{\cdot}053$). Further analyses showed that the psoriasis and PPP patient groups were quite similar regarding ECP levels in serum. In the PPP group the geometric mean ECP in serum (28·2 μg/l) was significantly higher than that of the control group (16·5 μg/l, $P = 0{\cdot}019$). A comparison of the geometric mean in the psoriasis group (24·3 μg/l) with that of the control group showed a tendency in the same direction ($P = 0{\cdot}098$). The individual values are shown in Figure 1.

Total number of circulating eosinophils

There was no significant difference between the geometric mean number of eosinophils in the psoriasis ($\bar{x} = 156 \times 10^6$/l, SE = 1·13, $n = 35$), PPP ($\bar{x} = 173 \times 10^6$/l, SE = 1·13, $n = 16$) and control group ($\bar{x} = 135 \times 10^6$/1, SE = 1·10, $n = 19$).

Correlation between ECP and total number of circulating eosinophils

A relation was found between the total number of circulating eosinophils and ECP in serum; in the psoriasis group $r = 0{\cdot}54$, $P < 0{\cdot}001$ and the PPP group $r = 0{\cdot}48$, $P < 0{\cdot}05$. No significant relation of this kind was seen in the control group.

ECP in suction-blister fluid

(a) There was no significant difference as regards the mean concentrations of ECP in blister fluid in controls ($\bar{x} = 6{\cdot}0$ μg/l) and in the uninvolved skin of patients with psoriasis ($\bar{x} = 4{\cdot}2$ μg/l).

(b) The ECP concentration in blister fluid from lesional psoriatic skin (median 18·1 μg/l) was significantly higher than in the blister fluid from normal control skin (median 6·1 μg/l, $P = 0{\cdot}012$) (Fig. 2).

(c) The concentration of ECP in blister fluid from lesional psoriatic skin was significantly higher than that in blister fluid from uninvolved skin in the same patient (mean difference = 18·2 μg/l, $P = 0{\cdot}03$) (Fig. 2).

(d) When the mean concentration of ECP in blister fluid from involved skin in active psoriasis ($\bar{x} = 31{\cdot}5$ μg/l) (progressing during the last weeks) was compared with that in blister fluid from lesions in more slowly progressing and/or stable psoriasis ($\bar{x} = 10{\cdot}8$ μg/l), a significant difference was found ($P = 0{\cdot}005$; Table 1).

ECP in skin biopsy specimens

The specimens from the psoriatic lesions could be divided into three groups with regard to the histological pattern (Table 2). ECP was present in all of the 15 specimens from lesional skin, but its location seemed to vary between the three histological groups. These groups differed from each other with regard to the presence of granulocytes, but all showed parakeratosis, with an absence of the granular layer and acanthosis. Activated eosinophils and secreted ECP, as evaluated by the presence of EG2, were observed in 11 of the 15 specimens.

TABLE 2. Number of eosinophils per section stained only with haematoxylin and eosin (H–E) and the degree (+ to + + + +, see text) of ECP-and EG2-immunoreactive sites in the immunohistochemically stained sections from lesional psoriasis skin. The same patient numbers as in Table 1 are used, a–c indicate biopsies from the same patient. Depending on the histological findings the specimens were divided into three groups (I–III)

	Patient No.	Number of eosinophils H–E stained	Degree of ECP immunoreactivity	Degree of EG2 immunoreactivity
Group I	6(a)	2	+ + + +	+ + +
	6(b)	2	+ + +	+ +
	10	7	+ + +	+ +
	13(a)	0	+ + + +	+ + +
	14(a)	1	+ + + +	+ + +
	14(b)	4	+ + + +	+ + +
	14(c)	1	+ + +	+ +
Group II	1	1	+ +	0
	2	0	+ +	+ +
	4	1	+	0
	7	0	+ + +	+ +
	12	2	+ +	+ +
	13(b)	0	+ +	+
Group III	5	4	+ +	0
	11	0	+	0

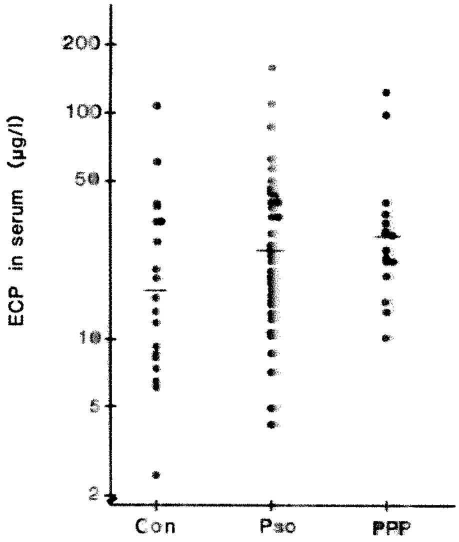

FIGURE 1. The individual ECP concentrations in serum in the healthy control group (Con), the psoriasis group (Pso) and the group of patients with pustulosis palmoplantaris (PPP). Horizontal bars indicate the geometric means.

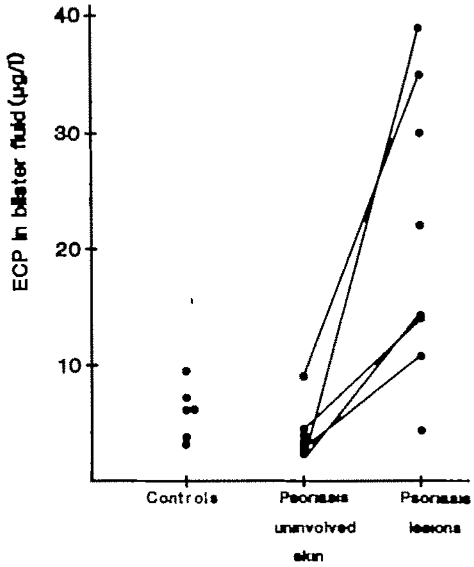

FIGURE 2. The individual ECP concentrations in blister fluid from healthy control skin and from uninvolved and lesional skin of psoriasis patients. The lines connect dots representing the same patients.

The first pattern (group I) was observed in seven of the 15 specimens. In the haematoxylin and eosin-stained sections there were small groups of granulocytes and microabscesses in and below the stratum corneum. Occasionally an eosinophil granulocyte could be seen in the stratum corneum. In the upper dermis there was a perivascular lymphohistiocytic infiltrate. Some neutrophils and, exceptionally, one or two eosinophils were observed in the upper part of the dermis.

The number of cells reactive to ECP antibody and EG2 antibody, and extracellular deposition of granuloproteins in each section were graded from 0 to +.+ + + (+ indicated just a few immunoreactive cells or aggregates of extracellular scattered ECP/EPX, + + meant five to 15 groups of extracellular ECP/EPX and a few immunoreactive cells, and + + + + indicated more than 10 immunoreactive cells and over 40 groups of extracellularly deposited ECP/EPX; see Table 2). ECP was observed in association with groups of granulocytes in the stratum corneum and in the upper third (Fig. 3) of the stratum spinosum, but usually not in the lower two-thirds of the epidermis. The precise extracellular location of the ECP in relation to the observed granulocytes could not be determined. ECP was also observed in the papillary and uppermost layers of the non-papillary dermis (Fig. 4), sometimes in relation to the lymphohistiocytic infiltrate. In this first group the amount of ECP detected was + + + to + + + +.

EG2 was observed in the same areas as ECP antibody but in small amounts, with + + to + + + reactions in each section. The number of eosinophils found at routine histological examination was always much smaller than the number of ECP-immunoreactive sites in the sections.

Patient No. 14 with an acute erythrodermic type of psoriasis and with pustulation, had the greatest deposition of ECP and of activated eosinophils in the biopsies of erythematous papules (Figs 5 and 6). In another biopsy of a pustule from this patient there were streaks of ECP-

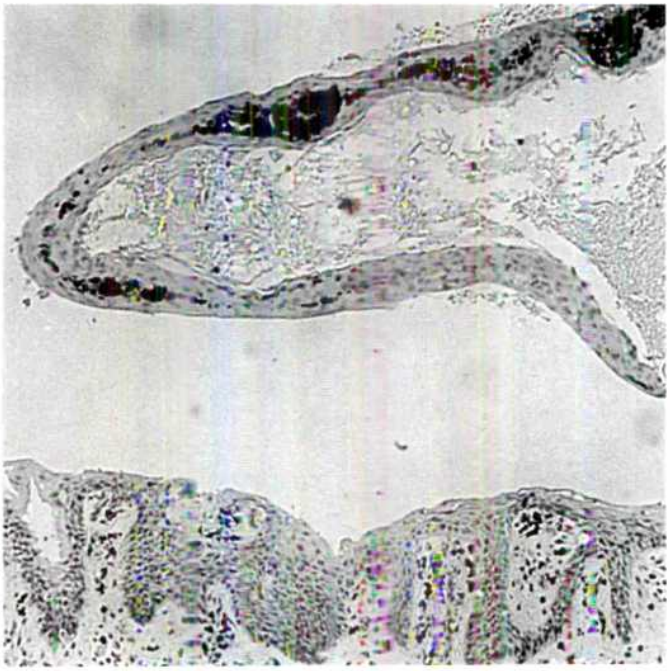

FIGURE 3. Biopsy No. 13a. ECP visualized with the PAP procedure counterstained with Harris' haematoxylin. Large amounts of ECP in stratum corneum. ECP is also deposited in dermis (see also Fig. 4).

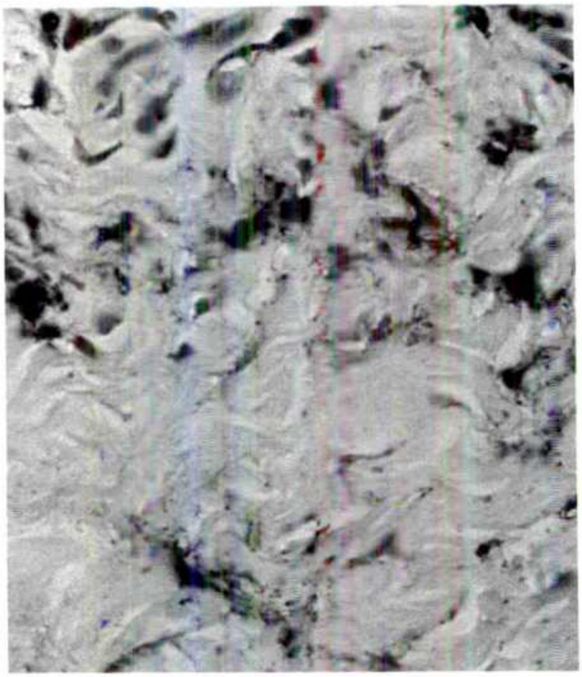

FIGURE 4. ECP immunoreactive cells and aggregates of extracellularly scattered ECP in the dermis (same specimen No. 13a as in Fig. 3) (× 6[illegible]).

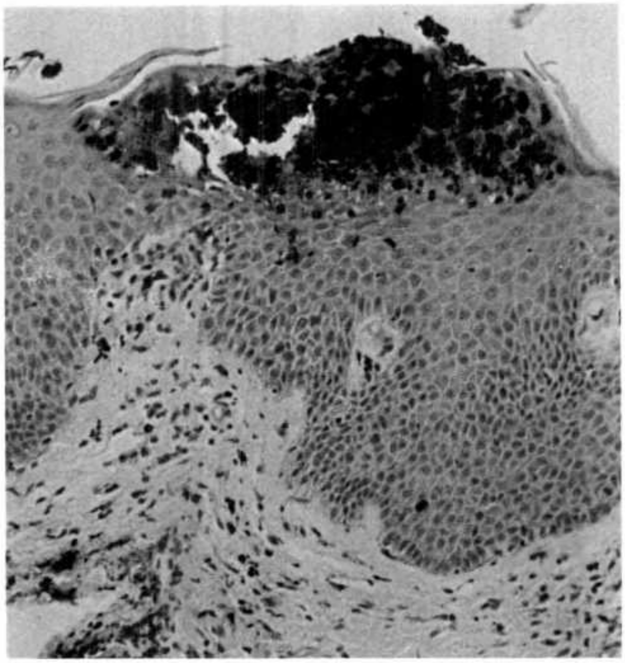

FIGURE 5. Specimen (No. 14b) from a fresh erythematous papule showing large amounts of ECP deposits in the upper part of the epidermis and in the papillary and uppermost layer of the non-papillary dermis. PAP and counter-staining Harris haematoxylin. (× 120).

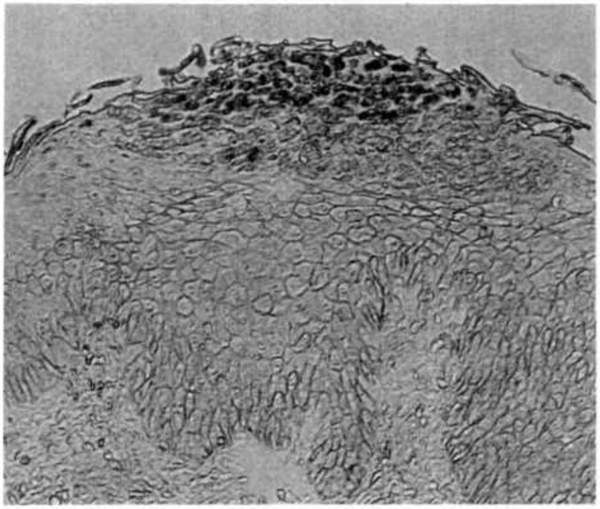

FIGURE 6. The same specimen (No. 14b) as in Fig. 5 stained with the APAAP procedure to visualize activated eosinophils and secreted ECP. EG2 antibodies are observed in the same areas as ECP antibodies but in smaller amounts (× 120).

positive staining through the epidermis from the papillary dermis to pustules in the stratum corneum (Fig. 7).

The second pattern (group II) observed in the haematoxylin and eosin-stained sections of six of the 15 specimens was characterized by a lack of granulocytes in the parakeratotic stratum corneum, and no microabscesses. No more than two eosinophils were observed per section. In the dermis there was a perivascular lymphohistiocytic infiltrate. The ECP reaction was + to + + + and was located in the parakeratotic stratum corneum and in the papillary and reticular dermis. In four of the six ECP-positive biopsies EG2-immunoreactive cells and secreted ECP/EPX were also observed (+ to + +).

A third pattern (group III) was noted in two of the 15 specimens. The stratum corneum was parakeratotic, but areas of orthokeratosis and a granular layer were also present. There were no granulocytes in the stratum corneum. In the dermis a few granulocytes were seen and one of the two patients also showed a few eosinophils. The ECP reaction was weakly positive (+ to + +) in these patients and the positive areas were only observed in the papillary and subpapillary parts of the dermis. In the specimens with pattern 3 no reaction to EG2 was found.

Specimens from uninvolved psoriatic skin and skin of healthy subjects

Neither ECP nor activated eosinophils, as evaluated by the presence of EG2, were found in the uninvolved skin from either the psoriasis patients or the healthy controls. No eosinophils were observed in the skin sections.

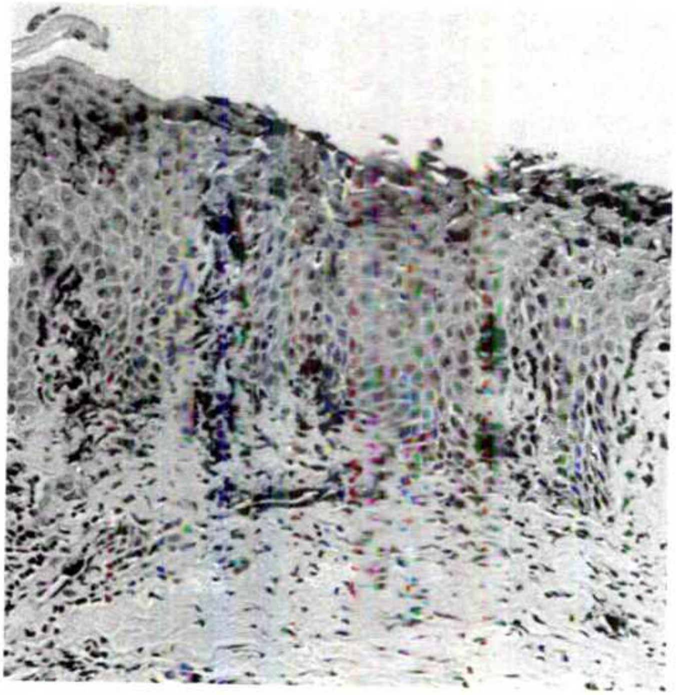

FIGURE 7. Specimen (No. 14a) from a macroscopically visible pustule demonstrating streaky pathways of ECP positive sites through the epidermis from the papillary dermis to the pustules in the stratum corneum. PAP and Harris' haematoxylin.

Relation of clinical findings to ECP in serum, blister fluid, and biopsy specimens

Neither the serum level of ECP nor the number of eosinophils in the blood, except in two patients with severe disease, were related to the clinical condition as judged by the degree of erythema, the number of pustules, scaling or skin area involved. One almost erythrodermic patient had 552 × 10^6/l eosinophils in the blood and the highest level of ECP in the serum (156 μg/l) and one PPP patient with over 700 pustules on the soles and palms had increased ECP in the serum (124 μg/l).

The highest concentrations of ECP in suction-blister fluid were found in blisters from patients with active and progressive psoriasis. It was not possible to examine the relationship between the amount of ECP in the sections and the age of the psoriatic lesion, as the exact age of the lesions was not always known. Patient 14, with the most acute psoriasis, had the largest amount of ECP in stratum corneum whereas patient 5 who had the most stable psoriasis, had no epidermal deposits of ECP.

There was no relation between the serum and the blister fluid ECP. Nor was there any relation between the serum ECP, the number of circulating eosinophils and the number of ECP-immunoreactive cells and the amount of extracellularly deposited ECP in the biopsy specimens.

DISCUSSION

In recent years it has become clear that eosinophil granulocytes are present in the tissues in various diseases[8,9,13] to a greater degree than may be evident from routine staining of biopsy specimens. Eosinophils that have degranulated and extracellular deposits of granule proteins are difficult to detect in routinely stained sections. The methods used in this study for detection of eosinophils and ECP are based on immunohistochemistry with the use of two types of antibodies: polyclonal ECP antibodies that react both with intracellular and extracellular ECP, and monoclonal EG2 antibodies, that react with ECP/EPX in activated eosinophils and secreted proteins. Their specificities have been checked in detail and reported.[5,9] ECP was observed in all specimens from psoriatic lesions (either as ECP immunoreactive cells or extracellular deposit of ECP without associations with cells) and at many more sites than where eosinophils were detected by haematoxylin and eosin staining. Although the number of biopsies was limited, the results indicate that more ECP is found in those specimens with large numbers of granulocytes in the upper part of the epidermis. In the dermis from uninvolved skin no eosinophils or ECP deposition were found. The mechanism for the migration of eosinophils towards the stratum corneum is unknown. However, the eosinophil chemotactic factors, PAF, C5a and LTB4, are all known to be present in psoriatic epidermis.[15,16] Several other factors such as eosinophil chemotactic factors (ECFs) and eosinophil stimulation promotor (ESP) have also been reported[4] to stimulate eosinophil migration, but their presence in psoriatic lesions has not been reported.

Activated eosinophils and secreted ECP/EPX were found in the same areas as ECP, detected by the polyclonal ECP antibodies, but with a weaker expression. This may indicate that only part of the eosinophils in the lesions were in fact activated, and that the process of activation probably takes place in the skin. However, differences in affinity and avidity are important in explaining the weaker expression of activated eosinophils. Granule proteins from eosinophils (ECP and/or MBP) have recently been demonstrated in the dermis of patients with urticaria using similar techniques.[17,18] Similarly, they have been found in Wells syndrome,[13] atopic dermatitis and in onchocerciasis.[8] The dermal deposits of the eosinophil granule protein were mainly extracellular and there were no epidermal deposits of eosinophil granule proteins.

The involvement of eosinophils in psoriasis was also studied by the measurement of ECP in suction blister fluid. In all the controls except one and in the uninvolved skin of the psoriasis patients, the concentration of ECP in the blister fluid was lower than in serum.

Blisters were produced both in patients with active psoriasis and in those with slowly progressive lesions. The mean ECP concentration in blister fluid from the lesions of active patients was about three times higher than in the blister fluid from uninvolved skin. It seems probable that most of the ECP is derived from a local increase in eosinophils, as judged by the presence of ECP in the epidermis and dermis in the biopsy specimens. Although there seemed to be no relation between the level of ECP in blister fluid and that in the serum, the possibility of increased leakage of circulating ECP through the papillary vessels cannot be ruled out.

The role played by eosinophils in inflammatory reactions is not yet established but it has been suggested that eosinophils may have a modulatory action.[4,19] ECP and other eosinophil-derived proteins may also have different biological effects at different concentrations. Studies on the effects of ECP on lymphocytes have indicated that it may increase suppressor cell activity.[20] It is conceivable that ECP in the concentrations observed in the blister fluid may influence the activity of lymphocytes both *in vivo* and *in vitro*.

Stimulated eosinophils can produce 15-HETE.[19] 15-HETE has been observed together with 12-HETE in psoriatic lesions. Both compounds were found in higher concentrations in the upper part of the lesions.[21] Injected 15-HETE has been reported to have a healing effect on psoriatic lesions.[22] Whether the eosinophils observed in dermal and epidermal lesional psoriatic skin may produce 15-HETE in biologically relevant amounts, e.g. to modulate the inflammatory reaction, is unknown, but this possibility cannot be excluded.

Eosinophil products and especially ECP have cytotoxic properties that may give rise to tissue damage, as seen in Wells syndrome, eosinophilic cystitis, inflammatory bowel diseases and Löffler's endomyocardial disease.[13] In this study we found increased amounts of eosinophils and ECP in psoriatic lesions and it seems likely that eosinophils are involved in the psoriatic process.

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The specificity and cellular origin of phenylethanolamine *N*-methyltransferase (PNMT)-like immunoreactivity in psoriatic skin

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SUMMARY

Phenylethanolamine *N*-methyltransferase (PNMT)-like immunoreactivity has been found in psoriatic skin and in this study, PNMT-like immunoreactivity was investigated in the involved and uninvolved skin of six patients with lichen planus and four patients with lichen simplex. No PNMT immunoreactivity was observed in these diseases. Studies were carried out using cultured fibroblasts from two patients with psoriasis from uninvolved and involved areas of skin and from two controls using antibodies to PNMT, as well as antibodies to the chemical messengers somatostatin, substance P, parathyroid hormone and peptide histidine isoleucine amide. No immunoreactivity to these substances was found, and fibroblasts are unlikely to be the cellular origin of the PNMT-like immunoreactivity as seen in psoriatic skin.

In a previous study,[1] phenylethanolamine *N*-methyltransferase (PNMT)-like immunoreactivity was found in the dermis and epidermis of the involved skin in psoriasis. The increase was 10-fold in involved skin as compared to that in the uninvolved skin, and normal skin contained no PNMT-like immunoreactivity. No immunoreactivity to the other catecholamine synthesizing enzymes (tyrosine hydroxylase, dopa-decarboxylase, and dopamine-β-hydroxylase) could be found, apart from tyrosine hydroxylase positive sympathetic nerves.[1]

In this investigation, the specificity of the PNMT-like immunoreactivity as found in psoriasis was evaluated and other dermatoses were investigated. Lichen planus and lichen simplex were chosen since they are both inflammatory and hyperkinetic disorders,[2–4] and in both the involved skin can be clearly distinguished from uninvolved areas. Immunofluorescence[5] was performed on thin sections of tissue using antibodies against PNMT and analysed using computer-assisted image-processing techniques.

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In our earlier study[1] the PNMT-like immunoreactivity was confined to cellular structures of the upper dermis. Since the cell type involved was unknown, but the oval shape of the immunoreactive profiles resembled fibroblasts, fibroblast cultures were also studied for immunoreactivity to PNMT. The chemical messengers somatostatin (SOM), substance P (SP), peptide histidine isoleucine amide (PHI), and parathyroid hormone (PTH) were investigated for screening purposes, since neuropeptides have been suggested to play an important role in psoriasis.[6–8] It should be noted that certain dermal factors also seem to be of importance for epidermal growth *in vitro*.[9,10]

METHODS

Subjects

Six patients with lichen planus and four patients with lichen simplex were investigated. Data concerning the patients are presented in Table 1. Sixteen patients with psoriasis and six healthy controls from the previous study[1] are included for comparison. The biopsies for fibroblast cultures were taken from both the involved and uninvolved skin of two untreated psoriatic patients (two males, 28 and 79 years old, duration of disease 0·5 and 30 years, respectively), and from two healthy controls (two males, 25 and 39 years old). No oral or topical treatment had been given to the patients in the 4 weeks prior to biopsy. Human and rat adrenal medulla were used as positive controls.

Antisera

Antibodies to PNMT,[11] SOM,[12] SP,[13] PHI,[14] and PTH were generated in rabbits, goats and chickens. The anti-PTH was a commercial antiserum from the Nichols Institute, Los Angeles, CA, U.S.A. The same batch of PNMT antiserum was used as in the earlier study[1] and normal serum or blocked antiserum was used for controls. The antisera used showed no cross-reactivity with other substances in the radioimmunological or immunohistochemical tests. However, cross-reactivity with known, untested substances, or with unknown proteins/polypeptides/

TABLE 1. Patient and control groups: clinical summary and PNMT-like immunoreactivity as characterized by computer-assisted image analysis

	Psoriasis*	Lichen planus	Lichen simplex	Control*
Clinical summary				
No. of patients	16	6	4	6
Sex (M/F)	9/7	3/3	2/2	3/3
Mean age and range (years)	43 (17–74)	42 (20–63)	50 (44–64)	30 (24–37)
Mean duration of disease and range (years)	14 (0·3–37)	1·5 (0·03–3·5)	10 (2–20)	—
Results				
PNMT-LI,† volume density (%; mean (SEM)), involved skin	4·52 (0·36)	0·00	0·00	—
PNMT-LI,† volume density (%; mean (SEM)), uninvolved skin	0·63 (0·003)	0·00	0·00	0·00

* Previously reported.[1]

† Phenylethanolamine *N*-methyltransferase-like immunoreactivity.

peptides present in the tissue sections, with a chemical composition similar to PNMT cannot be excluded and therefore the term 'PNMT-like immunoreactivity' has been used in the text.

Preparation of tissue

Punch biopsies (3 mm) were taken under local anaesthesia with lidocaine without epinephrine from the involved and uninvolved skin (minimum distance to involved skin 4 cm). The biopsies were immersed for 2 h in a solution of picric acid and formalin. The tissue was then rinsed for at least 24 h in 0·1 M Sörensen's buffer containing 10% sucrose, 0·01% NaN_3, and 0·02% Bacitracin (Sigma Chemical Co. St Louis, MI, U.S.A.), and 14 μm sections cut using a cryostat and processed for indirect immunohistochemistry as previously described.[1,15]

Fibroblast cultures

The fibroblast cultures were established from 3 mm punch biopsies. The cells were propagated in Earle's modified Eagle culture medium (EMEM, Flow Laboratories, Irvine, Scotland, U.K.) containing 10% human A serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml Fungizone (Gibco, Paisley, Scotland, U.K.) and 2 mM L-glutamine (Gibco) and used after nine passages. Final growth took place on coverslips that had been placed at the bottom of 60-mm Petri dishes. The fibroblasts were incubated in a humid atmosphere of 5% CO_2 and 95% air at 37°C.

Immunohistochemistry of sectioned biopsies

The sections were incubated, with the antiserum or control serum diluted 1:800 in 0·01 M phosphate-buffered saline (PBS), overnight at 4°C in a humid atmosphere, rinsed in PBS for 10 min with three changes, then incubated for 30 min at 37°C with fluorescein–isothiocyanate (FITC)-labelled anti-rabbit serum (Boehringer Mannheim Biochemicals) diluted 1:10, rinsed as before and mounted in glycerine/PBS (10:1) containing 0·1% *p*-phenylenediamine. As an additional control, certain sections were incubated only with the second antiserum. All antisera contained 0·3% Triton X-100. The sections were studied and photographed using a Zeiss fluorescence microscope equipped with a dark-field oil condenser and a HBO 200 high pressure mercury lamp with a 3 or 4 mm Schott BG12 or a KP500 as excitation filter and a Zeiss 50 or LP520 as barrier filter.

Immunohistochemistry of cultured fibroblasts

The medium was removed and each coverslip was covered with a solution of picric acid and formalin, then rinsed in 0·1 M Sörensen's buffer with sucrose, NaN_3, and Bacitracin (see above). The coverslips were then incubated, with the antisera diluted (PNMT: 1:800; SOM: 1:400; SP: 1:200; PHI: 1:400; PTH: 1:400; control sera: corresponding dilutions) in PBS, overnight at 4°C in a humid atmosphere, rinsed in PBS for 15 min, then incubated for 30 min at 37°C with FITC-labelled anti-goat, anti-chicken (Nordic Immunology), or anti-rabbit (Amersham International) serum diluted 1:10 and rinsed as described in the previous paragraph. All antisera contained 0·3% Triton X-100. The cultures were then studied using a Zeiss microscope.

Computer-assisted image processing

Representative sections from each lichen planus and lichen simplex patient were analyzed further by a computer-based image-processing system (IBAS, Zeiss/Kontron) using grey-scale high-resolution algorithms.[1,15,16]

RESULTS

Using immunohistochemistry, no immunoreactivity for the catecholamine-synthesizing enzyme PNMT could be found either in involved or in uninvolved skin from the patients with lichen planus and lichen simplex (Fig. 1a). This was confirmed by high-resolution image-processing (see Table 1). No fluorescence was found after incubation with control serum or with the second, FITC-labelled, antiserum alone.

There was no immunoreactivity to PNMT, SOM, SP, PHI, or PTH in cultured fibroblasts obtained from involved and uninvolved psoriatic skin or from normal controls.

DISCUSSION

The results from lichen planus and lichen simplex patients are in contrast to earlier observations on psoriasis,[1] where marked immunofluorescence was seen in the basal epidermis and upper dermis in 16 patients (Fig. 1b). The amount of PNMT-like immunoreactivity, as characterized by computer-assisted image analysis, was 10 times higher in the involved psoriatic skin as compared to that in the uninvolved skin. The uninvolved areas in psoriasis also showed PNMT-positive structures not seen in the skin from the six healthy volunteers (Table 1).[1]

The presence of PNMT-like immunoreactivity in psoriasis[1] could be regarded as a secondary phenomenon that is common to other proliferative and inflammatory skin disorders. However, in this study an absence of PNMT-like immunoreactivity was found in two dermatoses that

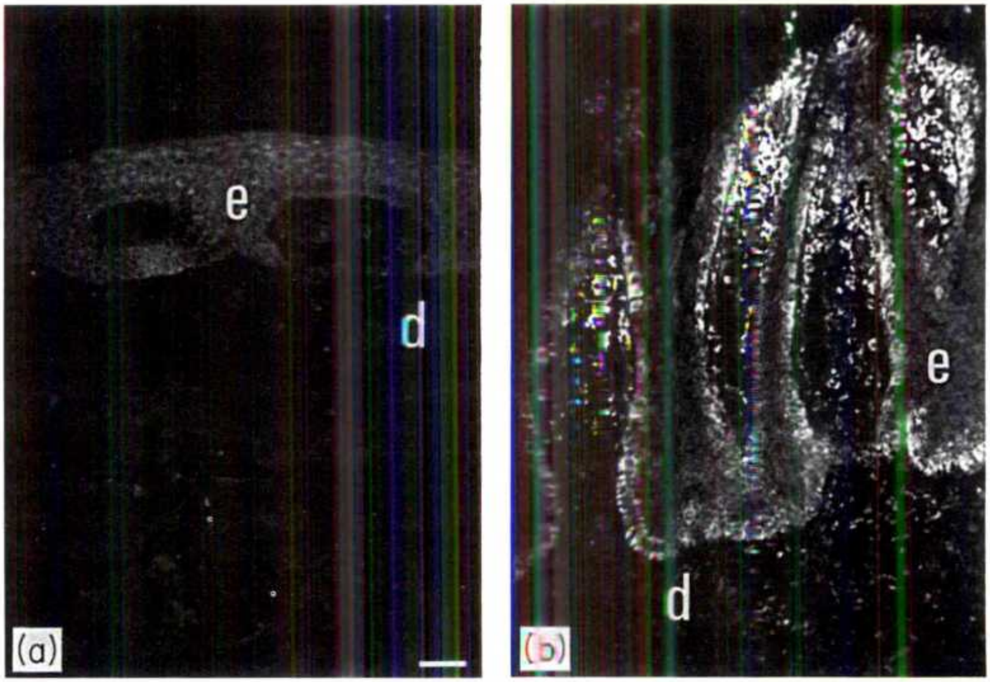

FIGURE 1. Immunofluorescence micrographs of involved skin from lichen planus (a) and psoriasis (b) after incubation with antiserum to PNMT. In (a), no immunoreaction is observed, whereas a large amount of PNMT-like immunoreactivity is seen in (b). d, dermis; e, epidermis. (a) and (b) have the same magnification. Bar indicates 50 µm.

have several features similar to those seen in psoriasis. This suggests that immunoreactivity to PNMT may be specific for psoriasis. A decrease of β-receptors was recently reported in involved psoriatic epidermis as compared to controls.[17] This suggests that the observed increase in PNMT-like immunoreactivity is secondary to a defect or a decrease of the total number of ligand-binding sites for epinephrine in the psoriatic epidermis. However, it has not yet been shown whether this decrease of β-receptors is specific for psoriasis.

In our previous study[1] PNMT-like immunoreactivity was confined to cellular structures in the upper dermis. The density of cellular structures was high, though the type of cell was unknown. However, the oval shape of the immunoreactive structures had some resemblance to fibroblasts and fibroblast cultures were therefore studied for their PNMT-like immunoreactivity. The fibroblast cultures obtained from involved or uninvolved psoriatic skin showed no PNMT-like immunoreactivity. This makes it unlikely that fibroblasts are responsible for the dermal pattern of PNMT-like immunoreactivity as seen in psoriasis. It has been suggested to us that the relevant cell type may correspond to the factor XIIIa$^+$ dermal dendrocyte that has recently been reported and which is increased in number in the upper dermis in lesions of psoriasis.[18,19]

The catecholamine pathway is enzymatically represented in the skin and dopa-decarboxylase, dopamine-β-hydroxylase and catechol-O-methyl-transferase have been reported.[20-22] It is possible that this catecholamine pathway is involved in a paracrine catecholamine secretory function in the epidermis and further studies are indicated to determine the importance of catecholamines and catecholamine enzymes in the pathogenesis of psoriasis.

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Are ACA and Scl 70 antibodies mutually exclusive?

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SUMMARY

Serological studies using indirect immunofluorescence, double immunodiffusion and immunoblot with recombinant Topo I and kinetochore antigens were performed in 180 patients with systemic scleroderma. In the sera of 10 patients, we found coexisting ACA and Scl 70 antibodies, and this was confirmed by all three methods. The relationship between the coexistence of these antibodies and the clinical features of the disease is not clear, although in all these cases there was pronounced vascular involvement.

The recognition of the antinuclear antibodies specific to systemic sclerosis (SSc): ACA and Scl 70,[1–3] is of special importance since these antibodies are found to be markers of different subsets of the disease.[1,2,4–9] ACA is a marker of a more localized form of SSc in which there are few cutaneous indurations,[7] and is referred to as the CREST syndrome (calcinosis, Raynaud, esophagus, sclerodactyly, telangiectasia). This was regarded as a benign variant of SSc but several cases have been reported with severe vascular and systemic involvement and some with a fatal outcome.[3,6,7,9] The frequency of detection of ACA varies from 25 to 96%.[1,2,4,7,9] In a previous study of 114 patients we found ACA in only 25% of those with the limited form of the disease, but in 52% of those with cutaneous indurations limited to the digits.[7] In a larger series of 140 patients with limited disease, ACA was found in 25·7% (unpublished data).

Scl 70 was initially described as an antibody characteristic of diffuse scleroderma.[1,2] The frequency of detection of Scl 70 in diffuse scleroderma ranges from 18 to 67%.[1,2,8–10] In a previous study of 107 SSc patients we found Scl 70 in about 50% of all cases and in 77% (30/39) of these with the diffuse variety.[8] In a larger series of 252 patients, Scl 70 was detected in 86·6% of those with the diffuse variety and in 51·4% of those with limited disease (unpublished data).

ACA and Scl 70 antibodies were regarded as mutually exclusive by us and others[8,9,11] and there has only been a single report of coexistence.[12,13] A recent review[11] stated: 'Although the immune response in scleroderma is fairly diverse it appears that patients do not produce

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autoantibodies to several antigens simultaneously as they do in lupus. The previously mentioned mutual exclusion in occurrence of anti-Topo I and anticentromere antigens is an example of this phenomenon.'

We report 10 cases of coexisting ACA and Scl 70 antibodies using indirect immunofluorescence (IIF), double immunodiffusion (DID) and immunoblot (IB) technique.

METHODS

One hundred and eighty patients with systemic sclerosis (SSc) were chosen for study. Three methods were employed for the detection of Scl 70 antibody and ACA: indirect immunofluorescence (IIF) on HEp-2 cells for both antibodies, double immunodiffusion (DID) for Scl 70, and immunoblot (IB) with recombinant kinetochore antigen for ACA and with recombinant Topo I for Scl 70.

Indirect immunofluorescence

The sera were screened on monkey oesophagus and, if positive, titrated on HEp-2 cells (Immco Diagnostic, Buffalo, NY, U.S.A.) starting with a dilution of 1/10. The conjugate used was goat anti-human IgG (Heintel, Vienna, Austria) with a fluorescein to protein ratio of 2·2, 16 U/ml, diluted to contain 1/8 U/ml.

Double immunodiffusion

This was performed according to the method of Tan *et al.*,[1] where the Scl 70 antigen was prepared from calf thymus according to the same authors, as modified and standardized by Kumar *et al.*[14]

Immunoblot

The IB method was performed with the use of recombinant Topoisomerase I and kinetochore antigens (Wistar Institute, Philadelphia, PA, U.S.A.). β-galactosidase fusion proteins were isolated from lysogens derived as previously described.[15,16] The Topo I fusion protein contained the carboxyterminal 109 amino acids of Topo I.

The kinetochore fusion protein has not been characterized except that its primary sequence differed from the antigen described by Earnshaw *et al.*[17] The antigens were separated according to Laemmli[18] and electroblotted in nitrocellulose.[19]

The reactions with patients' sera (diluted 1 : 100) and alkaline phosphatase-labelled goat anti-human IgG (Promega, Madison, WI, U.S.A.) were visualized by NBT and BCIP.

RESULTS

The results of immunofluorescence studies, DID and IB are shown in Table 1. Ten patients were found to be positive for both Scl 70 and ACA. In all 10 cases, the Scl 70 pattern was predominant in IIF, and in the majority of the cases an ACA pattern could only be detected at higher dilutions (1/80–1/320). Only in patient 5 were both patterns seen in lower dilutions, and the dividing cells showed characteristic features either of Scl 70, or ACA, or both (Fig. 1a–c).

In some cases, the ACA pattern was only seen in some interphase cells (Fig. 2), while dividing cells showed the fluorescence of the Scl 70 type. Scl 70 was confirmed in all cases by DID and IB using recombinant Topo I. IB was positive for kinetochore antigen also in all cases (Fig. 3).

The clinical details of the 10 patients found to be positive for both ACA and Scl 70 are shown

TABLE 1. Results of immunological studies with the use of three techniques: indirect immunofluorescence, double immunodiffusion and immunoblot with recombinant antigens Topo I and kinetochore

	IIF on HEp-2 cells						
	Pattern typical of Scl 70 (titre)	Pattern typical of ACA in single			DID positive for Scl 70	Immunoblot (recombinant antigens)	
Patient		Interphase cells	Dividing cells	Titre		Topo I	Kinetochore
1 G.T.	160	+	+	80	+	weak +	+ +
2 D.C.	1280	+	–	160	+	+	weak +
3 C.M.	640	–	–	0	+	+	+
4 G.S.	20	–	–	0	weak +	+ +	+
5 Z.C.	640	+*	+	5120	+	weak +	+
6 J.F.	2560	+	+	320	+	+	+
7 J.S.	2560	+	–	160	+	+ +	+
8 S.P.	2560	+	+	320	+	+	weak +
9 A.R.	640	+	–	160	+	+	weak +
10 G.Z.	640	+	–	160	+	+ +	weak +

* In this case the ACA pattern was present in the majority of the cells.

in Table 2. All were female, (mean age 38·8 years) one being an 8-year-old girl. Limited scleroderma was recognized in three, and diffuse cutaneous indurations were present in six. In one patient the changes were atypical, with syringomyelia-like indurated areas on the hands, and oedamatous changes on the extremities and the face. There was also marked atrophy of both sides of the face. The 8-year-old girl had Raynaud's phenomenon and sclerodactyly, with only minimal cutaneous induration. In all patients there was pronounced vascular involvement, with telangiectasia of the skin on the face in eight and disseminated numerous telangiectases on the trunk and upper extremities in five of the patients. Calcinosis was found in five cases, four of them having diffuse SSc. Visceral changes were present in nine patients irrespective of the variety of SSc and course of the disease; the only exception was the child. There was a rapidly progressive course in three patients, who had the diffuse variety, and in two of them there was kidney and muscle involvement. One patient died from renal failure. There was no correlation between the titres of Scl 70 antibodies in IIF and the course of the disease. The patient with the most severe disease had a relatively low titre of Scl 70 antibody. The highest titres of Scl 70 were found in patients with a slowly progressive course, but with the diffuse variety of the disease.

In seven cases, the IF ACA pattern was seen in single cells at the dilutions 1:80–1:320, i.e. their titre was lower than that of Scl 70. In one case with limited scleroderma, in which the ACA titre was 5120 (higher than the Scl 70 titre), a typical ACA pattern could be seen in numerous cells, both interphase and dividing. In two cases, ACA was not shown by IIF, but detected by immunoblot for kinetochore.

In two cases of diffuse SSc and in one of limited disease with minimal cutaneous indurations IB reaction for kinetochore was faint, and both IIF and DID were positive for Scl 70.

A case of limited SSc with calcinosis and profuse telangiectasia, gave a very strong reaction with IB for Topo I, although the titre of Scl 70 in IIF was very low and DID was only weakly positive. The IB reaction for kinetochore was positive, but we were unable to find an ACA pattern with IIF. It is probable that the levels of both antibodies were very low.

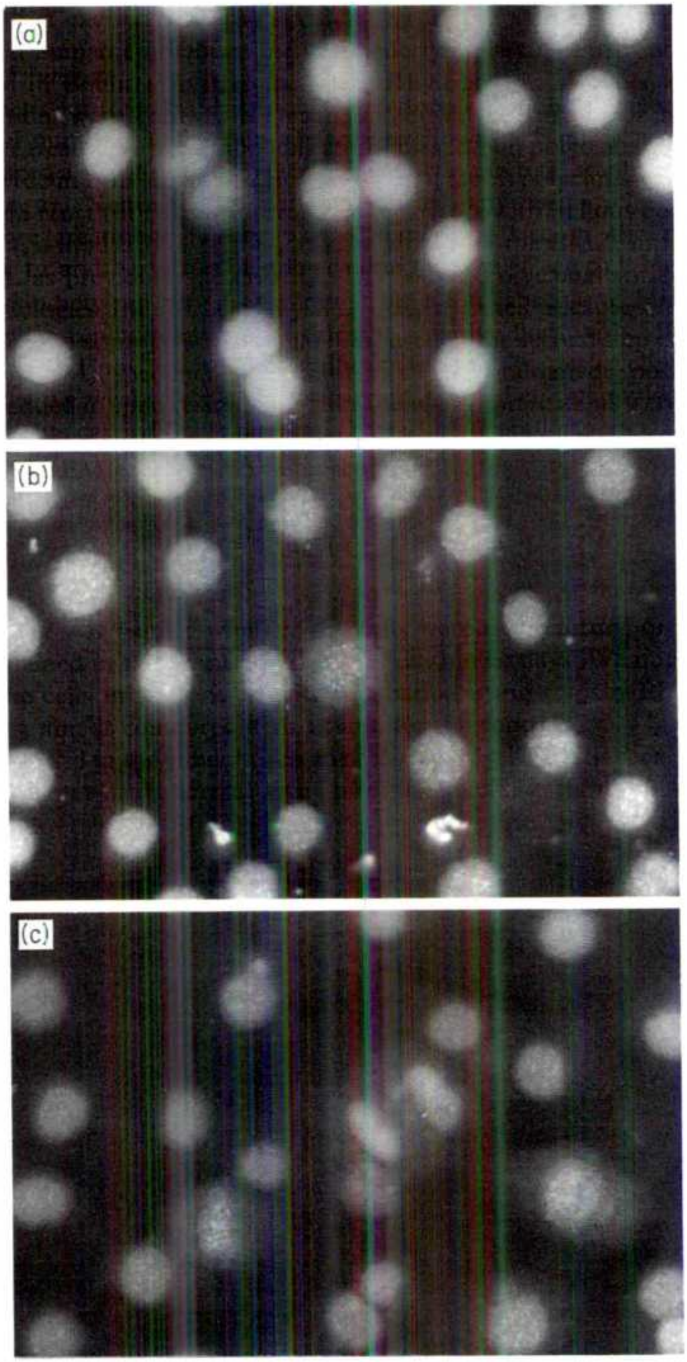

FIGURE 1. Patient 5. Indirect immunofluorescence on HEp 2 cells. (a) Serum diluted 1:10. In the interphase cells the pattern is typical of Scl 70. To the left is a mitotic cell typical of Scl 70. (b) The same serum diluted 1:20. In most cells, there is interference showing the Scl 70 and ACA patterns. In the centre is a mitotic cell typical of ACA. (c) The same serum. Interference showing both patterns is present. Centre right: mitosis typical of Scl 70, and to the left, mitosis typical of ACA.

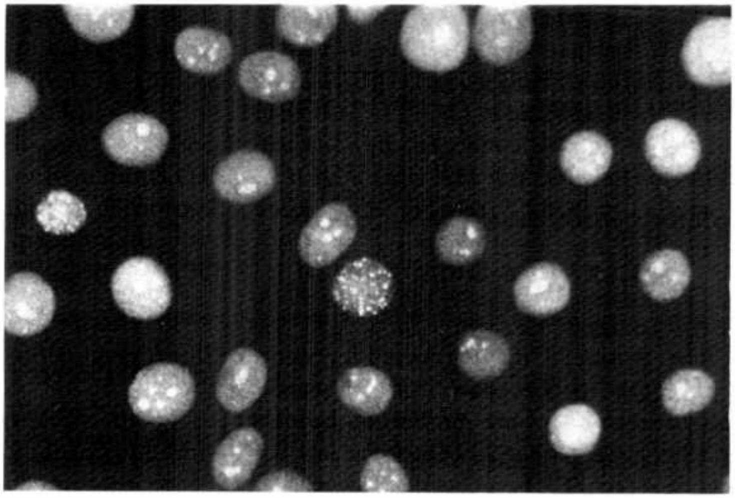

FIGURE 2. Patient 8. Indirect immunofluorescence on HEp-2 cells. Serum diluted 1:160. ACA pattern in interphase cells (in the centre, and to the left), other cells display typical Scl 70 pattern.

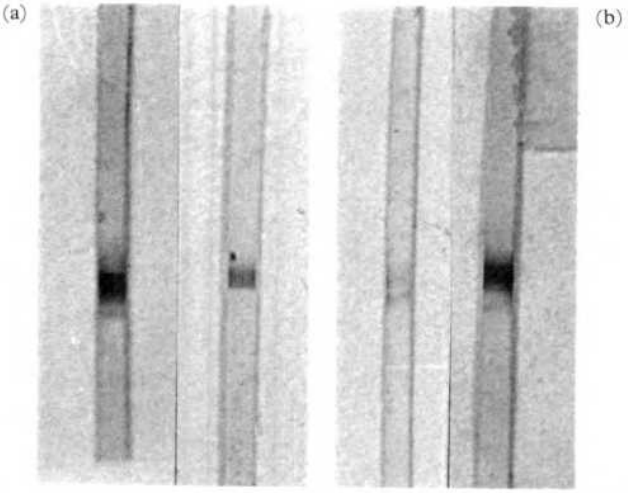

FIGURE 3. Immunoblot of two SSc sera with recombinant Topoisomerase I and kinetochore antigens. (a) strong Topo I and weaker kinetochore staining (patient 4) (b) weak Topo I and strong kinetochore staining (patient 1).

DISCUSSION

With the use of three techniques, IIF, DID and IB, it was possible to detect the coexistence of two types of antibodies specific to SSc, Scl 70 and ACA in 10 of 180 cases (5·5%). The unexpected initial finding of kinetochore antibody in a case of diffuse scleroderma positive for Scl 70 antibody in DID and IIF, led us to re-evaluate the IIF patterns on HEp2 cells. By careful examination of numerous cells in different serum dilutions we could discern in single-interphase cells the pattern characteristic of ACA, in addition to prevalent Scl 70 staining. In the next four cases, the presence of ACA was detected initially by IB, and again in some cells the IIF pattern

TABLE 2. Clinical characteristics of patients in whom Scl 70 and ACA antibodies were disclosed

	Duration (years) of		Visceral involvement*						Cutaneous involvement		Vascular involvement					
											Telangiectasia			Ulcerations		
Patient Sex/Age	Raynaud	Indurations	E	H	L	K	M	Hughes score	Limited s	Diffuse s	Face	Disseminated	Calcinosis	Finger-tip	In other location	Course of the disease
1 G.T. F/40	5	3·5	+	+	+	+	+	18		+	+	−	+	+	+	Rapid; exitus
2 D.C. F/32	11	7	+	−	+	−	−	9		+	+	+	+	+	+	Moderate
3 C.M. F/40	11	8	+	+	−	+	+	15		+	+	−	−	+	−	Rapidly progressive
4 O.S. F/60	6	4	+	[illegible]	[illegible]	[illegible]	[illegible]	3	+		+	+	+	+	−	Slowly progressive
5 Z.G. F/46	20	7	+	+	+	−	−	11	+		+	−	−	+	−	Slowly progressive
6 J.F. F/39	21	13	+	+	+	−	−	11	atypical		+	+	+	+	−	Slowly progressive
7 I.S. F/37	19	6	+	+	+	−	−	12		+	+	+	+	−	−	Slowly progressive
8 S.P. F/55	14	5	+	+	−	−	−	9		+	+	+	−	+	−	Slowly progressive
9 A.R. F/8	2	?	−	−	−	−	−	1	+		−	−	−	−	−	?
10 G.Z. F/32	From early childhood	2	+	−	−	−	+	9		+	−	−	−	+	−	Rapidly progressive

*E, esophageous; H, heart; L, lung; K, kidney; M, muscle.

was characteristic of ACA. Later ACA was detected by IIF, though this pattern was in all cases masked by Scl 70 antibodies. To detect the coexistence of ACA on HEp-2 cells, it was necessary to evaluate cells at both metaphase and interphase in different serum dilutions. The masking of ACA in IIF by the prevalence of Scl 70 is probably why this coexistence has not been detected by others and the presence of ACA should always be checked with IB. Scl 70 has a very characteristic pattern on HEp-2 cells, and can be detected by IIF, and then confirmed by DID. Thus, both these antibodies are disclosed on HEp-2 cells. In our cases of coexistence, the prevalent antibody was Scl 70, and in only one patient was the ACA pattern already visible in numerous interphase and dividing cells in lower dilutions, whereas in other cases it was seen only in higher dilutions, starting from 1:80, and in single cells. It appears that in cases of coexistence of Scl 70 antibody and ACA the latter are present in lower titres than in cases positive for ACA alone. The previous report of the coexistence of Scl 70 and ACA in 2 cases also had very low titres of ACA.[12]

To demonstrate the masking effect of Scl 70 *in vitro*, we mixed, in various proportions, serum with a high titre of ACA (1:5120) and serum containing Scl 70 antibody with a titre of 1:640. The ACA pattern was initially masked by Scl 70, although this was of a lower titre, and the ACA pattern started to appear in dilutions of 1:80 of the mixture. Both the titre of ACA and the proportion of serum containing ACA must be much higher than that of Scl 70 antibody in order to visualize ACA at lower dilutions. ACA may be missed due to the masking effect of Scl 70 and the coexistence of ACA and Scl 70 antibody appears to be more frequent than previously supposed.

The clinical features of our cases differed, and the cases were either of the diffuse or limited scleroderma type. All the cases had vascular involvement, and half had calcinosis of the skin. Only in an unusual case of an 8-year-old girl was there limited scleroderma with minimal cutaneous indurations confined to the digits; features characteristic of ACA. However in this girl, the titre of Scl 70 antibody in IIF was 640, and DID was positive for Scl 70.

In addition to these 10 cases of coexistence of ACA and Scl 70 antibodies we had two cases of ACA coexisting with other antibodies (Pm-Scl and La/Ro), one of Scl 70 with spindle apparatus antibody and one of Scl 70 with NSPI.[20] The coexistence of antibodies in SSc is much less frequent than in SLE, but does occur, and the two antibodies, highly characteristic of SSc, Scl 70 and ACA, do not seem to be mutually exclusive.

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Investigation of skin by ultraviolet remittance spectroscopy

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SUMMARY

Remittance spectroscopy in the ultraviolet spectral region was used to investigate human skin *in vivo*. Untreated healthy skin showed a minimum remittance at 280 nm wavelength, corresponding to the keratin absorption band. At shorter wavelengths the remittance mostly originated from light scattering in the stratum corneum and at longer wavelengths subcorneal structures had increasing influence. The spectral remittance reflected variations in pigmentation most sensitively in the UVA region. Hydration of the skin yielded a hypsochromic shift of the keratin absorption band due to changes in polarity and proton concentration in the environment of the chromophores. Remittance spectroscopy allows detection of topically applied drugs, whose penetration into the skin can be evaluated from the time-dependence of the remittance.

Information about cutaneous optics can be obtained non-invasively *in vivo* by studying spectral remittance or, via use of photothermal methods,[1] spectral absorption. The former technique has been used mainly in the visible spectral region for the objective determination of skin colour, especially for quantifying erythema and pigmentation induced by exposure to ultraviolet (UV) radiation. Recent studies have demonstrated that it is possible to differentiate quantitatively between the erythemal response and the simultaneous tanning of the skin. Advances in instrumentation allow avoidance of any contact with the skin area being tested with the use of remote viewing heads.[2–6]

Skin is a turbid tissue, which shows a strongly wavelength-dependent penetration depth for optical radiation.[7] In the UV spectral region, the penetration depth of light is small due to high absorption in stratum corneum and epidermis, the major absorbers being melanin, urocanic acid and aromatic amino acid residues. Only a small portion of the incident light is remitted (diffusively reflected) by these layers.

Since the original work by Hasselbalch,[8] many studies in the UV spectral region of reflection by and transmission through excised samples of stratum corneum and epidermis have been reported. With a collimated incident beam of light, widely used in these experiments, the measurements yield information about direct transmission (in the direction of the incident beam), total transmission and diffuse reflectance.[9] Due to diffuse forward-scattering, which is the dominant scattering mechanism in stratum corneum and epidermis,[10] the total transmission exceeds the direct transmission. Recently a mathematical model has been proposed by Diffey[11] which allows evaluation of the intrinsic optical properties of a turbid sample, e.g. the spectral coefficients of absorption, back-scattering and forward-scattering, from such measurements.

We report *in vivo* measurements of spectral remittance in the UV spectral region aimed at the investigation of optical properties of normal and moisturized Caucasian skin. We also report on measurements determining the penetration behaviour of UV-absorbing drugs after topical application to the skin. Recent results obtained with other techniques, namely fluorescence spectroscopy[12] and photoacoustic spectroscopy,[13] have demonstrated that spectroscopic *in vivo* techniques can be successfully used in this field of pharmacological research.

METHODS

The instrument used for the *in vivo* remittance measurements was a commercially available UV/VIS-spectrometer (Lambda 15, Perkin-Elmer, D-7770 Überlingen) with a spectral range from 200–900 nm. The measuring beam was transmitted via UV-transparent, flexible fibre optics to the viewing head. The monochromatic, collimated beam was directed perpendicular to the skin. The illuminated skin area had a diameter of about 10 mm. To avoid the spectral reflection from the surface being detected, the receiving fibres, which carried the remitted light back to the spectrometer, were mounted at an angle of 45° to the skin surface. The fibres were positioned such that the same area was both illuminated and viewed. In the spectrometer a photomultiplier was used as a broad-band detector for the remitted light.

The remittance of the skin was related to that of a remittance standard ($BaSO_4$ for the visible and Teflon for the UV-region) to take into account the spectral sensitivity of the spectrometer.

The *in vivo* measurements were performed on healthy, fair-skinned Caucasian test subjects, aged 23–28 years, on the inner aspects of the forearms. The results presented here are mean

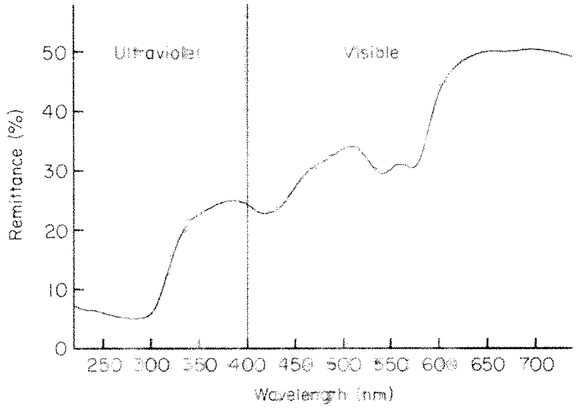

FIGURE 1. Mean spectral remittance, obtained *in vivo* at the inner aspects of the forearms of three fair-skinned test subjects.

values of spectral remittance obtained with three volunteers. For the measurement on moisturized stratum corneum the skin was hydrated with a water-soaked swab for 10 min. For the diffusion experiment, 6 μl of the formulation containing the drug to be detected were uniformly distributed over a skin area of 4 × 5 cm², forming a screening layer of 3 μm mean thickness on the surface of the skin. In between the times of measurement, the test areas were covered by Finn-chambers to prevent external removal of the drug by absorption or abrasion by clothing. To avoid an increase in horny layer hydration by occlusion, perforated Finn-chambers were used.

RESULTS

The spectral remittance of fair-skinned Caucasian subjects is depicted in Figure 1. The local minima of remittance at wavelengths 415, 540 and 572 nm corresponded to the absorption bands

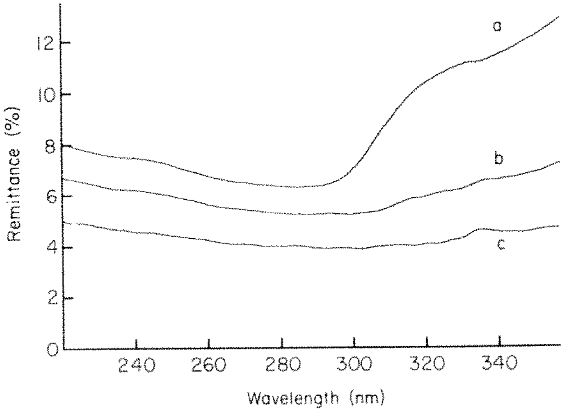

FIGURE 2. *In vivo* remittance spectra of melanin-containing skin, obtained at the inner aspect of the forearm. a, Weakly pigmented skin, b, moderately pigmented skin, c, strongly pigmented skin.

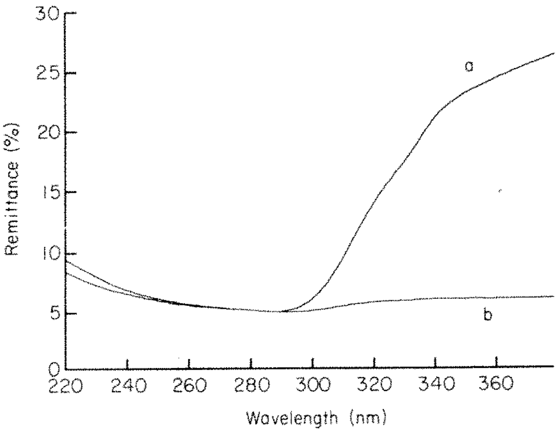

FIGURE 3. *In vitro* remittance spectra of excised, fair stratum corneum. a, Stratum corneum terminated by a diffuse reflector; b, stratum corneum terminated by a black absorber.

of haemoglobin, demonstrating the remittance in the visible spectral region to be due to light scattering in the vascularized dermis. Irradiation with red light yields remittance values as high as 40–60%, depending on skin pigmentation.

The melanin content of the epidermis can be most sensitively deduced from UVA (315–400 nm) remittance. In Figure 2 spectra are plotted as obtained with weakly, moderately and strongly pigmented Caucasian skin. Curve a of Figure 2 steeply increases with increasing wavelength at the long-wave side of the keratin absorption band, the maximum of which coincides with the shallow minimum of remittance near 280 nm. With increasing pigmentation the spectral remittance flattens out (curve c of Fig. 2), as melanin, whose absorption depends only weakly on wavelength, becomes the major absorber.

In the short-wave UV, below about 300 nm, the spectral remittance originates from the light scattering in the stratum corneum, even if the pigmentation is weak. To demonstrate this, we measured the remittance of isolated stratum corneum. The samples were prepared from cadaverous skin. After detachment of dermis and epidermis, the horny layer was obtained by trypsin treatment.[14] Figure 3 shows the spectral remittance of isolated stratum corneum, measured with the sample terminated by teflon as diffuse reflector (curve a) and by a black absorber (curve b). In the short-wave UV both curves show no significant differences, but above 300 nm, the stratum corneum becomes translucent and the curves diverge. Applied to *in vivo* measurements, this means that light is transmitted to and remitted from deeper epidermal layers only at wavelengths above 300 nm.

Hydration of living skin alters the water content of the stratum corneum, especially of its outermost layers. The spectral remission of fair Caucasian skin before and after treatment with a water-soaked swab for 10 min is shown in Figure 4. At the long-wave side of the keratin absorption band, between 290 and 340 nm, hydration clearly increases the spectral remittance. While no significant changes are observed at longer wavelengths, a weak increase is detectable over all the short-wave UV. In parallel to the spectral remittance, the pH-value was measured at the skin surface by use of a pH-meter (Radiometer PHM 80) with calomel electrode and flat glass electrode as reference and probe electrode, respectively. Due to the hydration the pH-value was shifted from 5·8 to 6·8.

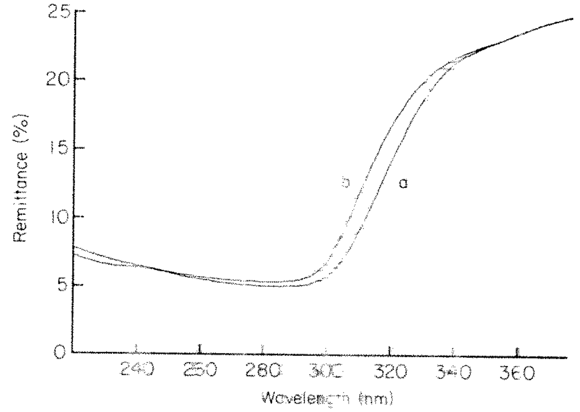

FIGURE 4. Influence of skin hydration on spectral remittance. a, Untreated skin; b, hydrated skin.

Topical application of a thin vehicle film containing a light-absorbing drug lowers the spectral remittance of the skin as both the incident and the remitted light have to pass the film. To obtain information about the absorption spectrum of the drug, uninfluenced by the optical properties of the applied vehicle film, the measurement has to be performed by comparing the spectral remittance of treated skin to that of skin treated with the unloaded vehicle. Figure 5 shows remittance spectra obtained with fair-skinned subjects after treatment of the inner aspect of the forearm with a film of water-in-oil (W/O) emulsion and with a film of W/O-emulsion containing 2% of the sunscreening agent Uvinul® T150. The difference between the curves shows the *in vivo* absorption spectrum of the agent under test. Maximum absorption is observed at a wavelength of about 320 nm (Fig. 6). After topical application to the skin surface, the UV-

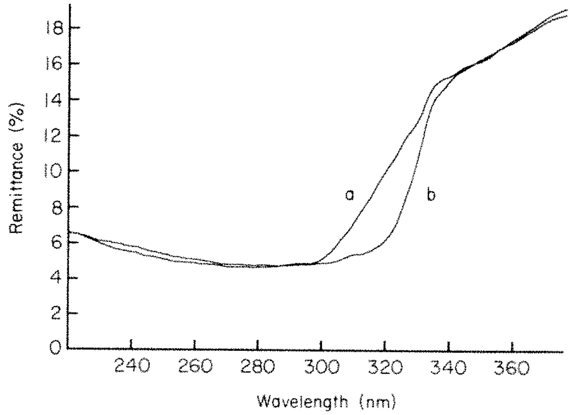

FIGURE 5. Detection of drugs topically applied to the skin. a, Skin treated with an unloaded W/O-emulsion; b, skin treated with a W/O-emulsion containing 2% Uvinul® T150.

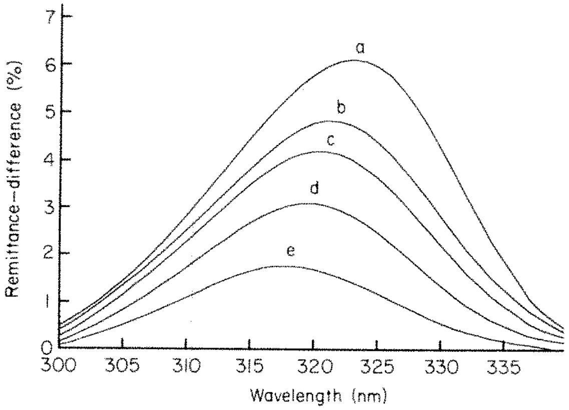

FIGURE 6. Difference in remittance signals between skin treated with an unloaded vehicle and skin treated with the same vehicle containing 2% Uvinul® T150, showing the *in vivo* absorption spectrum of the drug. a, Immediately after application; b, 3 h after application; c, 6 h after application; d, 10 h after application; e, 24 h after application.

filter penetrates into the skin by diffusion. In the stratum corneum, the main diffusion barrier of the body, the penetration proceeds very slowly and in general, permeation by molecules of low molecular weight requires many hours.[13] The diffusion of sunscreen into the skin reduces the shading of the remittance. Figure 6 shows the differences in spectral remittance, measured at different times after topical application of the unloaded emulsion as placebo and the emulsion containing the sunscreen.

DISCUSSION

Since the original work of Sheard and Brown,[15] Bode,[16] Edwards and Duntley,[17] the techniques for the investigation of *in vivo* remittance of human skin at visible wavelengths have improved. However, to date physicians have mostly relied on their visual assessment to quantify erythema or pigmentation. Even in spectral regions invisible to the human eye, remittance spectroscopy has not found widespread use, although the first applications of *in vivo* remittance spectroscopy with UV-wavelengths was reported as early as 1955.

In the UV region, the penetration depth of light is restricted to the epidermis or, at wavelengths below 300 nm, to its outermost layer, the stratum corneum. This is mainly due to the high absorption of light in these layers; the influence of light scattering to the penetration depth is comparatively small. The spectral remittance, which is proportional to the ratio of scattering and absorption coefficient,[7] is also small. The spectral remittance can serve as a sensitive detector of variations in either the absorption or scattering coefficient, if the coefficient not being studied can be treated as a constant or weakly varying parameter.

With the instrumentation used here fluorescence could not be detected. Skin fluorescence contributes up to 20% of the remittance,[18] especially in the UVC region. The investigated drug Uvinul® T150 shows fluorescence with an emission peak at 420 nm, and the decrease of remittance observed after application of the drug would probably be more pronounced if the remitted fluorescence light could be filtered out. Nevertheless, the time course of remittance due to the penetration of a light-absorbing drug into the skin is only gradually influenced by the fluorescence effect.

These results demonstrate that remittance spectroscopy can be used *in vivo* for the investigation of the optical properties of human skin. Only at the long-wave limit of the UV region does light scattering in the papillary dermis contribute to the remittance of fair skin, as shown by considering the influence of the haemoglobin absorption on the spectral remittance. Transmission spectra of blood show that haemoglobin absorbs much more strongly at 415 nm than at 540 and 572 nm,[7] whereas the *in vivo* spectral remittance of fair skin shows a local minimum at 415 nm which is relatively weaker than those at 540 and 572 nm (Fig. 1). At moderate or high levels of epidermal melanin content, the 415 nm band is no longer detectable.

In the short-wave UV region, below a wavelength of about 300 nm, the penetration depth of light is smaller than or comparable to the thickness of the stratum corneum (Fig. 3). In the UVA region the spectral remittance yields information about the epidermis. The increase in melanin content, which lowers the remittance at all UV wavelengths (Fig. 2), considerably reduces the penetration depth of light in the epidermis, so that in moderately or highly pigmented skin only the outermost epidermal layers contribute to the spectral remittance.

The stratum corneum is capable of taking up a considerable amount of water and even though water does not absorb ultraviolet or visible light, the optical properties of stratum corneum depends on the water content. The measurement of spectral transmission through excised samples has demonstrated that the keratin absorption band, which with dry samples is centred around 280 nm, is shifted to shorter wavelengths.[19] This hypsochromic shift was clearly detected by our *in vivo* remittance measurements (Fig. 4), and may be attributed to changes in polarity and proton concentration in the environment of the chromophores.[20,21] The hypsochromic shift of the keratinic absorption band in moisturized stratum corneum lowers the photoprotection of the underlying viable tissue against 290–340 nm UV radiation. In the shortwave UV region, below 290 nm, there is no significant variation of spectral remittance dependent on water content, and the effects of the gradual increase of scattering and attenuation with increasing water content, observed by transmission measurements on excised samples,[6] probably cancel each other out.

Remittance spectroscopy is a useful tool for *in vivo* detection of topically applied light-absorbing drugs. It allows the determination of drug distribution within the penetration depth of the incident light, which in the UV spectral region is restricted to stratum corneum and epidermis. After topical application, the drug shades the skin and this lowers the remittance. When released from the vehicle, the drug penetrates into the stratum corneum, diffuses into the epidermis and finally reaches the dermis, from which it may be absorbed into the blood capillaries. As the drug is transported into deeper skin layers, the effect on the remittance signal, which at UV-wavelengths results from scattering of light in the stratum corneum and epidermis, decreases and the remittance approaches that of untreated skin (Fig. 6). At 320 nm, the wavelength of maximum absorption of the sunscreening agent Uvinul® T150, the remittance originates from the stratum corneum and the superficial epidermal layers. The time dependence of remittance therefore reflects variations of drug distribution in these areas. A weak shift of the absorption maximum to shorter wavelengths is then seen as the drug continues to penetrate into the skin. Thus, a quantitative description of the pattern of remittance after topical application of a light absorbing drug can be obtained by taking into account the optical properties of untreated skin and the variations due to the time-dependent distribution of the light absorbing drug.

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Dermatitis due to intravesical mitomycin C: a delayed-type hypersensitivity reaction?

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SUMMARY

Mitomycin C is an alkylating agent, used by intravesical instillation to treat carcinoma of the bladder. Repeated instillations can induce cystitis and an eczematous eruption affecting the palms, soles and face. If these effects are due to delayed hypersensitivity with sensitization to mitomycin C occurring in the bladder wall, it should be possible to demonstrate antigen-presenting cells in the bladder wall and positive patch tests to the drug. Using an immuno-alkaline phosphatase method we have identified $CD1^+$ cells in bladder epithelium and submucosa and have demonstrated Birbeck granules in a few cells. In further support of our hypothesis it was also possible to demonstrate delayed type hypersensitivity in 13 out of 26 patients who had received mitomycin instillations by applying the allergen as a patch test. These results indicate that the eczematous eruption in this group of patients is most likely a hypersensitivity reaction and that it may be mediated transvesically.

Mitomycin C is an amino-quinone alkylating agent of molecular weight 334 Da used as an intravesical instillation to treat superficial bladder cancer. The surface layers of the bladder are exposed to therapeutic concentrations and very little of the drug is absorbed.[1] The side-effects of this treatment, occurring after repeated instillations, include vesical irritation and, less commonly, a rash involving the palms, soles, chest and face. The bladder symptoms have previously been attributed to chemical irritation[2] and the rash to a contact dermatitis following contamination of skin with urine containing mitomycin C.[3] However, the areas of involvement, which include the soles, make it unlikely that contamination of the skin accounts for the rash in all the cases. Evidence against an irritant or chemical cystitis was provided in a previous report of this symptom complex in which bladder histology, seen in biopsies taken soon after the onset of symptoms, revealed an inflammatory cell infiltrate with numerous eosinophils suggesting an immunological reaction.[4]

In this paper we have examined the hypothesis that the cystitis and dermatitis which may

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follow mitomycin C instillations are not the result of 'chemical irritation' but of a delayed hypersensitivity reaction to mitomycin, mediated transvesically. This was postulated when similar symptoms were described in a patient who was shown to have a positive patch test to mitomycin C.[5] For the hypothesis to stand it was necessary to establish two important points: whether there are any antigen-presenting cells in the human bladder (as there appears to be no published data on this subject), and whether patients with the cystitis–dermatitis complex have been sensitized to mitomycin C. Preliminary studies have indicated the presence of $CD1^{+}$ cells in the bladder epithelium and epicutaneous patch testing has demonstrated mitomycin hypersensitivity.[6]

METHODS

Patients

Seventy-five patients with primary superficial bladder cancer were studied. After their tumours were resected they were treated with bladder instillations of 0·1% mitomycin C (30 mg in 30 ml water) for 1 h; the mixture of mitomycin C and urine was then voided *per urethram*. The number of instillations varied from 0 to 10 and their frequency from 1 week to 3 months apart. From this group of patients separate subgroups of 26 and 10 were selected for patch testing and bladder biopsy respectively and were compared with controls.

Patch tests

Twenty-six of the mitomycin C-treated patients, including two who had previously had a rash, were patch tested to the European standard battery of common allergens and to two concentrations (0·01% and 0·001%) of freshly reconstituted mitomycin C in yellow soft paraffin. The tests were applied to the back and read at 48 and 96 h.[7] Ten normal volunteers were also tested to the two mitomycin concentrations.

TABLE 1. Details of bladder biopsies: bladder biopsies from males with benign prostatic hypertrophy

Patient no.	Clinical details	Number of mitomycin C instillations	Electron microscopy of Birbeck granules	Monoclonal antibodies						α Feto-protein
				[illegible]	CD8	CD4	CD22	M3	CD1	
1	Bacterial cystitis	0	NP	+ +	+ +	+ +	+	+ +	–	–
3		0	No	+ +	+ +	+ +	–	+	–	–
4		0	No	+ +	+ +	+ +	–	–	–	–
7		0	No	– –	–	–	–	–	–	–
8	Bacterial cystitis	0	NP	+	+ +	+ +	+ +	+ +	+ +	–
9		0	NP	+	+	+	–	–	+	+
10		0	No	+	+	+	–	+	–	–
11		0	NP	+	+	+	–	+	–	–

Cell counts + + = > 20 cells per low power (× 10) field.
+ = 1–20 cells per low power (× 10) field.
– = No staining.
NP, Not performed.

IgE estimations

An IgE estimation, using a double-antibody radioimmunoassay[8] was performed on all patients patch tested. None gave a history of atopic disease.

Identification of immunocompetent cells

Bladder biopsies. Biopsies were obtained endoscopically or at the time of open surgery from normal-looking mucosa. They were taken from 10 patients who had previously been treated for bladder cancer (seven male and three female, age range 53–87 years); two of them (Tables 1 and 2) had had the cystitis–dermatitis symptom complex. Eight control biopsies were obtained under similar conditions from males (age range 55–86 years) undergoing prostatectomy for benign disease. Specimens were processed for histopathology, immunohistology and electron microscopy.

Immunohistology. Cryostat sections (8 μm) were fixed in acetone for 10 min, washed with 0·001 mM Tris-buffered saline, incubated with primary antibody overnight at 4°C (Table 3), and stained by the immunoalkaline phosphatase (AP) method.[9] The conjugates were applied at room temperature for 40 min in a humid chamber. Sections were counterstained with haemalum. At least six pairs of serial sections were taken 40 μm apart. As a control, 20% normal rabbit serum in Tris-buffered saline or an irrelevant control (α feto-protein) was substituted for the first monoclonal antibody. All sections were examined 'blind'.

Electron microscopy. Bladder biopsy specimens from eight patients (Tables 1 and 2) were fixed in 3% glutaraldehyde in cacodylate buffer 0·1 M pH 7·4, washed in cacodylate buffer and post-fixed in 1% osmium tetroxide. The specimens were dehydrated in ethanol and embedded in

TABLE 2. Details of bladder biopsies: bladder biopsies from patients on mitomycin C trial for bladder cancer

Patient no.	Clinical details	Number of mitomycin C instillations	Electron microscopy of Birbeck granules	Monoclonal antibodies						α Feto-protein
				231	CD8	CD4	CD22	M3	CD1	
2		6	Yes	+	+ +	+ +	+ +	+	+	−
6		2	No	+	−	−	−	−	+	−
12	Rash and vesical irritation	5	NP	+	+ +	+	−	−	+	−
13		1	NP	+	+	+	−	−	+	−
15		0	NP	+	+ +	+	−	−	+	−
16		1	NP	+	+	+	−	+	−	−
17		0	NP	+	+	+	−	−	+	−
18	Rash and vesical irritation	4	NP	+	+	+	+	−	+ +	−
19		0	No	+	−	+	−	−	+	−
20	Cystectomy	3	Yes	+	+ +	+ +	+ +	+	+	−

Cell counts + + = > 20 cells per low power (× 10) field.
+ = 1–20 cells per low power (× 10) field.
− = No staining.

NP, Not performed.

TABLE 3. Antisera and their reactivity

Primary antisera	Origin	Immunoglobulin subclass	Antigen	Reactivity	Working dilution
Dako T6 M721	Dakopatts*	IgG2a	CD1	Langerhans cell/common thymocyte	1/200
Dako PAN B M708	Dakopatts	IgG2b	CD22	B cells	1/20
Anti Leu 2a	Becton Dickinson†	IgG1	CD8	Cytotoxic/suppressor T cell	1/100
Anti Leu 3a	Becton Dickinson†	IgG1	CD4	Helper/inducer T cells	1/100
Anti Leu M3	Becton Dickinson†	IgG2a	NK	Monocyte macrophage	1/100
DA6:231	Dr K.Guy‡	IgG1	NK	HLA -DP, -DQ, -DR	1/100
Anti α Feto-protein	Dr V.Van Heyningen	IgG1	NK	α Fetoprotein	1/40

NK, Not known.

*, Dako Ltd, 22 The Arcade, High Wycombe, U.K.

†, Diagnostic Systems, Becton Dickinson U.K. Ltd., Cowley, Oxford, U.K.

‡, Cytogenetics Unit, Western Infirmary, Edinburgh, U.K.

araldite. Ultra-thin sections were cut using a LKB Ultratome III, stained with uranyl acetate and lead citrate and examined with a Phillips EM 301. Twenty-eight blocks were cut and an average of three grids per block examined.

RESULTS

Patients

Fifteen of 75 patients receiving more than one instillation of mitomycin C developed dysuria, frequency and haematuria. Four of these cases also developed a palmar, foot and facial rash which occurred within 48 hours of treatment and persisted for several days. These problems were never seen after the first instillation but only after the second or subsequent instillations, the cystitis sometimes becoming so severe that the instillations had to be stopped.

Patch tests

Strongly positive patch tests (+ +) to 0·01% mitomycin C were seen at 48 and 96 h in both patients who had developed cystitis and a skin eruption. They did not react to the 0·001% mixture. Of the remaining 24 patients, 13 had negative (0) patch tests to both concentrations, seven had a positive (+) response to 0·01% only and four had a positive (+) response to both 0·01 and 0·001%. All 10 healthy volunteers had negative results to both concentrations.

IgE estimations

IgE levels were normal in 22 patients (upper limit of normal: 200 kU/l). Two individuals with markedly raised levels of 800 and 1000 kU/l respectively had negative patch tests and the other patient with a high level (650 kU/l) had a positive patch test to 0·01% mitomycin C. Judged by raised IgE levels the incidence of atopy in this group was 12%.

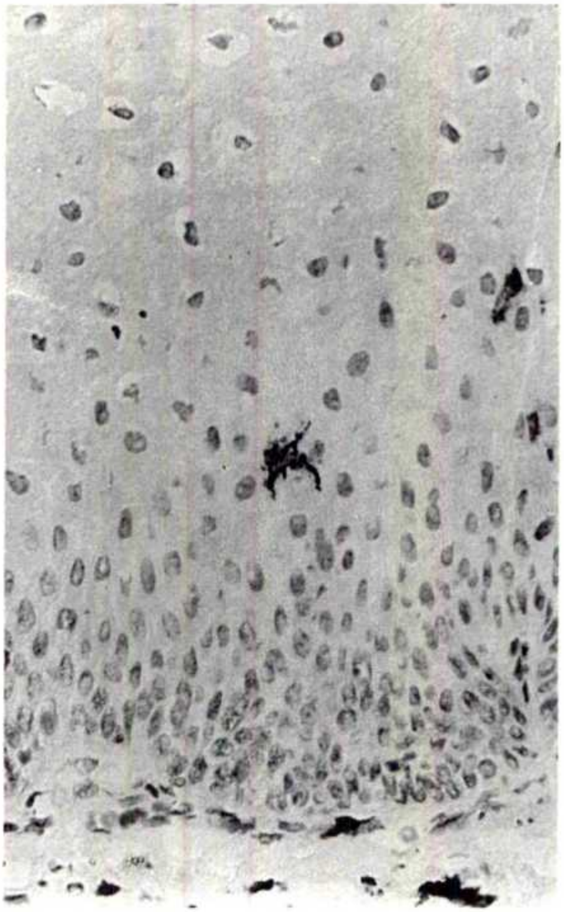

FIGURE 1. Photomicrograph of CD1^{+} dendritic cell in bladder epithelium (× 320).

Immunohistology

Only those cells with well delineated cell membranes were counted; the results are summarized in Tables 1 and 2. The cells of special interest were those staining for CD1; most of these cells had dendrites (Fig. 1). These were identified in two of the control specimens and in nine out of 10 specimens from patients with a history of bladder cancer. Most CD1^{+} cells were identified in the submucosa because the epithelium from several biopsies had been lost in processing, but in three cases (two with a history of bladder cancer) they were found in areas of adherent epithelium. Sub-mucosal cells positive for class II MHC antigens were found in all biopsies and T lymphocytes of both helper and suppressor phenotype were seen in 15 specimens.

B lymphocytes staining with CD22 and monocyte/macrophages staining with Leu M3 were found in about half of the specimens. They were prominent in the two patients who had had recent bacterial cystitis.

Electron microscopy

Despite an intensive search only two specimens were found to have occasional cells which contained Birbeck granules. Both specimens were from patients with a history of bladder cancer.

DISCUSSION

Some patients treated with repeated intravesical mitomycin C develop symptoms of vesical irritation and an eczematous eruption. The time course and increasing severity of the symptoms and signs suggest a cell-mediated delayed-type hypersensitivity reaction rather than immediate-type hypersensitivity. In support of this were the normal IgE levels noted in 12 out of 13 patients with positive patch tests. Strong evidence for delayed type hypersensitivity was provided by performing patch tests on an affected individual; a positive patch test was produced using 0·001% mitomycin in yellow soft paraffin.[5] In the present study both individuals whose skin had reacted to the instillation developed positive patch tests to 0·01% mitomycin. They had negative reactions to 0·001% mitomycin, but the results cannot be explained as an irritant reaction because all controls showed no reaction. The 11 weak responders (+) had all received at least four instillations and presumably, might have been candidates for vesical and skin symptoms had the instillations been continued.

It is a novel idea that sensitization, manifesting itself as a delayed-hypersensitivity reaction in the bladder and in the skin, could be induced across the bladder wall. We have demonstrated that some patients have developed cutaneous reactivity and that the necessary apparatus to mount this, i.e. antigen-presenting cells in the bladder wall, exists. We have not proved a mechanism for inducing sensitization nor explained the distribution of the eczema but it should be remembered that we have no good explanation for the distribution of other eczemas e.g flexural involvement in atopic eczema.

Mucosal surfaces are not a common site for sensitization, but it may occur in the mouth from anise oil[10], with metal fillings, or on the glans penis, from rubber condoms. Fisher[11] comments that the dermatitis associated with oral mucosal sensitization is usually symmetrical and eczematous. The gastro-intestinal mucosa has special properties and guinea-pigs, given a hapten orally prior to attempted cutaneous sensitization, developed tolerance.[12] More recently Lowney[13] demonstrated in humans that attempted buccal sensitization with DNCB resulted in half the subjects becoming sensitized and half becoming tolerant.

The process of sensitization requires cells capable of presenting antigens to T lymphocytes. Langerhans cells fulfill this role in the skin and are found in varying numbers in skin from different regions of the body.[14] Langerhans cells are also found in mucosal surfaces such as in the mouth[15,16] and in the buccal cavity their numbers are increased near squamous cell carcinomata.[17] Some authors believe that the size of the Langerhans cell population is directly related to keratinization,[18] but an inverse relationship has also been described.[15]

Langerhans cells have been found in the bladder mucosa of vitamin A-deficient Wistar rats[19] but we are not aware of any previous report of Langerhans cells in the normal human bladder. Dendritic cells staining for HLA-DR have been observed in the bladder wall (J.P.Sloane, personal communication). Langerhans cells were, however, found at post mortem in the bladder epthelium of a man who died of widespread malignant histiocytosis X.[20]

We have identified CD1$^+$ dendritic cells in the bladder epithelium and submucosa. We did not carry out double staining, but examination of adjacent sections suggest that the CD1$^+$ cells also expressed class II MHC molecules (DA 6·231$^+$). We also demonstrated Birbeck granules in a few cells from the same biopsies and it seems likely that they were in $CD1/231^+$ cells. The relative paucity of cells containing Birbeck granules may be explained in two ways. Firstly, few epithelial cells were seen because the epithelium was lost from many of the biopsies. Secondly the situation may be analogous to that in normal skin where dermal Langerhans cells are rarely identified because they seldom contain Birbeck granules. Dermal indeterminate cells are $CD1^+$ and most likely related to Langerhans cells.[21]

Sensitization through the skin is thought to depend on its many Langerhans cells. We found small numbers of $CD1^+$ cells in the normal bladder, but some of our patients with a history of bladder cancer had many more $CD1^+$ cells and some contained Birbeck granules as seen in typical Langerhans cells. The finding of Langerhans cells in mucosal surfaces has been associated with squamous metaplasia or neoplasia,[17] and it is possible that these changes in the bladder mucosa would render the surface capable of supporting the induction of contact hypersensitivity. Exposure of the mucosa to mitomycin could then, and only then, lead to a mitomycin-specific contact hypersensitivity. Once developed this sensitization would be systemically expressed and would be detected by positive patch tests.

We therefore postulate that Langerhans cells, present in the epithelium and submucosa of bladders in patients with a history of bladder cancer, are capable of hapten or antigen-presentation to T lymphocytes and, in this setting, probably play a key role in the induction of sensitization. Animal experiments (e.g. attempts to sensitize normal and vitamin A deficient rats by instillation of dinitrochlorobenzene into the bladder) could add support to our hypothesis or implicate alternative mechanisms such as the passage of antigens across the bladder wall and their presentation to the immune system by antigen presenting cells in the blood. We are not aware of any work on the subject.

If our hypothesis is correct, it raises the possibility that other allergens excreted in the urine could also induce sensitization. This may be of particular interest in other painful, non-malignant bladder conditions such as interstitial cystitis. This is a disease of obscure aetiology, characterized by a heavy infiltrate of eosinophils, mast cells and lymphocytes, which may indicate an immunological reaction. The cellular infiltrate has been noted[22,23] but it has not been possible to postulate a mechanism.

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Treatment of psoriatic arthritis by extracorporeal photochemotherapy

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SUMMARY

Five patients with long-standing seronegative arthritis resistant to conventional therapy and who also had psoriasis of the skin were treated with photopheresis. This mode of treatment combines a lymphocyte-enrichment procedure with 8-methoxypsoralen-photochemotherapy. There was a marked *in vitro* effect on treated lymphocytes, with a reduction of viability, proliferation and mitogen response. There was a slight to moderate clinical improvement in four of the five patients with regard to the strength of grip, swelling, pain, morning stiffness, the dosage of non-steroidal anti-rheumatic drugs and the radiographical changes. The skin lesions did not respond to photopheresis. Short-term side-effects were minimal and consisted of a temporary sensitivity of the eyes to light and signs of circulatory dysregulation immediately after treatment. This experimental therapy has some measurable but not dramatic effect in improving psoriatic arthropathy.

Psoriatic arthropathy is a distinct inflammatory joint disease which occurs in about 5–7% of patients with psoriasis.[1] The clinical features of this disease have been reviewed extensively[2,3] and the classification of patients with psoriatic arthritis into subgroups according to the pattern of joint involvement appears to have limited prognostic value. Approximately 10–20% of patients with psoriatic arthropathy may progress to a mutilating or ankylosing arthritis.[4] The pathogenesis of the condition is uncertain but immunological factors are involved.[5] Apart from a number of more subtle changes [6], particularly in humoral immunity[7], shifts in the ratio of the T4/T8 (helper/suppressor) phenotype of peripheral lymphocytes have repeatedly been reported in patients with psoriasis.[8–12] The effectiveness of immunosuppressive drugs such as cyclosporin A is suggestive of an involvement of for cell-mediated immune factors in its pathogenesis.[13] A genetic predisposition for the development of psoriasis as well as for joint involvement with an affinity to certain HLA-phenotypes has been observed.[14]

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Treatment of psoriatic arthritis has remained unsatisfactory and the administration of non-steroidal, anti-inflammatory drugs may be sufficient to control pain and inflammation in the milder forms. However, in progressive disease, more aggressive forms of therapy are employed, including hydroxychloroquine,[15] retinoids,[16,17] and cytotoxic agents,[18] in particular, methotrexate.[19] Whole-body psoralen photochemotherapy (PUVA) may produce an improvement.[20]

Recently, extracorporeal photochemotherapy or photopheresis was introduced for reducing inflammatory or neoplastic proliferation of lymphocytes. This treatment has been shown to be effective and safe with only minor side-effects in a large group of patients with cutaneous T-cell lymphomas.[21] Extracorporeal photochemotherapy involves a combination of a lymphocyte-enrichment procedure and photochemotherapy using 8-methoxypsoralen (8-MOP) with subsequent re-infusion of the cells. It may have an effect on expanding T-cell clones in benign diseases.[22] More recent investigations suggest a clone-limited response to extracorporeal photochemotherapy by demonstrating the inhibition of anti-skin allograft immunity by infusions with syngeneic photoinactivated effector lymphocytes.[23] As psoriasis and, to a certain extent, psoriatic arthritis respond to PUVA and beneficial effects are sometimes noted following a plasma exchange,[24,25] it seemed reasonable to treat patients with psoriatic arthritis with photopheresis. We report on a pilot study of five patients.

METHODS

Patients

Five patients (three males, two females; age range 26–58 years) with longstanding arthritis particularly involving the small joints and who were seronegative for rheumatoid arthritis were included in the study. All had typical skin lesions of psoriasis. Radiographic changes of joint disease were found in three of the patients. All five had received various forms of conventional treatment with little or no effect and four required non-steroidal anti-rheumatic drugs at the beginning of photopheresis treatment. Informed consent was obtained and the study was approved by the ethical committee of the University of Vienna.

Procedure

Extracorporeal photochemotherapy was performed using the UV-AR® instrument provided by Therakos® Company (King of Prussia, PA, U.S.A.). Two hours prior to treatment the patients received oral 8-methoxypsoralen (Oxsoralen®, Elder, Bryan, OH, U.S.A.) in a dosage of 0·6–0·8 mg/kg body weight. A lymphocyte concentrate containing 10^9–5×10^9 cells was collected using a conventional centrifuge apheresis procedure. To minimize the circulatory side-effects paediatric centrifuge bowls (Hemonetics®, Braintree, MA) were used. The total volume of blood extracorporeally processed in one treatment session varied according to the patient's haematologic parameters (primarily the haematocrit) and ranged from 1800 to 3500 ml. During the course of one single treatment 240 ml of pure lymphocyte concentrate and 200 ml of plasma were removed for further processing. Differences between patients and minimal variations in machine operation resulted in slightly variable lymphocyte counts and haematocrit values.

The lymphocyte concentrate was then exposed to UVA irradiation for 4·5 h after 200 ml of 8-MOP containing plasma had been added. The UVA dose of 122 J/cm^2 corresponds to a calculated dosage of 1–2 J/cm^2 for the lymphocyte when estimating a haematocrit of 5%. This dose was determined from a standard curve using monoclonal antibodies against 8-MOP in altered DNA.[26] The treated blood cells were then re-infused to over a period of 30–60 min.

Treatment

The patients had treatment on two consecutive days with a treatment-free period of 4 weeks. The relevant disease parameters that were recorded included morning stiffness, spontaneous pain, swelling of an indicator joint, grip strength and the radiographic changes (if present) as well as any change in the dosage of non-steroidal anti-rheumatic drugs.[27] The pain was quantified by questioning the patient with a four-grade scale (absent, light, moderate, severe). Measurement of joint swelling was performed by using stencils on the most severely affected interdigital joints. Grip strength was evaluated for both hands by squeezing a rubber balloon and was measured in mm/Hg. The haematological and biochemical parameters were constantly reviewed.

Evaluation of the clinical disease parameters was performed by an independent investigator. The extent of skin involvement in each patient was assessed and a skin score was determined by multiplication of the degree of skin changes (0, normal skin; 1, minimal erythema, papules; 2, strong erythema, scaling; 3, infiltrated plaques with scales or crusts; 4; massive inflammation, fissures) with the percentage of the body area involved. This procedure represents an adaptation of a method suggested by Edelson *et al.* to evaluate skin involvement in cutaneous T-cell lymphoma.[21] Treatment with photopheresis was continued either until a significant improvement of two or more of the clinical parameters was reached and maintained for more than 8 weeks, or until a change of concomitant therapy became necessary due to a deterioration of the patient's condition.

At the beginning of the study, the serum level of 8-methoxypsoralen was measured in each patient by means of high pressure liquid chromatography (HPLC) and a minimum level of 50 ng/ml was considered to be the least effective value.

Laboratory studies

To evaluate the reaction of the lymphocytes to the treatment, blood from all patients was processed at the first session but without being re-infused. After this mock treatment the following investigations were performed:

Lymphocyte viability. The percentage of viable lymphocytes in culture was determined by means of the trypan blue dye-exclusion test on day 1, 3 and 7 after treatment, with lymphocyte populations that had been exposed to UVA radiation for 60, 120, 180, and 270 min. This corresponds to 0·33, 0·88, 1·33 and 2·00 J/cm^2 of UVA radiation affecting the average target lymphocyte at a haematocrit of 5% in the concentrate. The same procedure was performed with a subpopulation that had been incubated with 8-MOP but not exposed to UVA.

Lymphocyte proliferation. The uptake of [^{3}H]-thymidine in lymphocyte cultures was measured in the absence and in the presence of a conventional mitogen (phytohaemagglutinin). Samples of cells which had undergone the combined treatment (8-MOP plus UVA) were compared to samples incubated with 8-MOP only.

RESULTS

Laboratory investigations

A significant reduction in the percentage of viable lymphocytes was noted after photopheresis. This reduction was directly proportional to the UVA dose with a time of 270 min of therapeutic UVA irradiation resulting in a 40–70% decrease of absolute viability 7 days after treatment. In

TABLE 1. Photopheresis in psoriatic arthritis. Lymphocyte viability (percentage of viable lymphocytes)

		Day after treatment		
Patient		1	3	7
B.K.	Pretreatment*	100	98	98
	After UVA treatment (h):			
	1	100	97	85
	2	100	97	82
	3	100	90	60
	4·5	100	86	22
W.R.	Pretreatment	100	99	96
	After UVA treatment (h):			
	1	100	97	92
	2	100	95	93
	3	100	79	60
	4·5	100	80	53
K.M.	Pretreatment	100	98	98
	After UVA treatment (h):			
	1	99	98	97
	2	99	92	96
	3	99	85	77
	4·5	99	77	59
D.H.	Pretreatment	99	99	99
	After UVA treatment (h):			
	1	99	98	87
	2	99	98	87
	3	99	98	79
	4·5	99	97	57
S.M.	Pretreatment	99	99	99
	After UVA treatment (h):			
	1	99	99	93
	2	99	99	93
	3	99	98	78
	4·5	99	95	60

* These cells had been exposed to 8-MOP *in vivo* but were not irradiated and also maintained in culture up to 7 days.

contrast, incubation with 8-MOP alone led to a 1–4% reduction of viable cells after the 7 days observation period (Table 1). A marked reduction of lymphocyte proliferation and mitogen response *in vitro* as measured by the [^{3}H]-thymidine incorporation rate was also found. The average counts before and after irradiation under spontaneous and stimulated conditions are given in Table 2.

Clinical response

All five patients had received various forms of treatment including gold salts, D-penicillamine and PUVA with short-lasting or unsatisfactory results. The total duration of photopheresis

TABLE 2. Photopheresis in psoriatic arthritis. Lymphocyte proliferation (incorporation of [^{3}H]-thymidine*)

Patient	Pretreatment ($x \pm s$)	Post-treatment ($x \pm s$)	Pretreatment (+PHA) ($x \pm s$)	Post-treatment (+PHA) ($x \pm s$)
K.M	2585 ± 1021	517 ± 139	120363 ± 9747	18615 ± 2874
D.H.	11915 ± 5045	5535 ± 1538	46318 ± 14608	10636 ± 2033
S.M.	1765 ± 356	117 ± 85	51820 ± 12497	1300 ± 458
W.R.	3218 ± 1701	1348 ± 189	197848 ± 24046	3262 ± 662
B.K.	17508 ± 4680	477 ± 238	56809 ± 4692	25156 ± 2073

* Values given are average counts per minute of five samples.

treatment was between 20 and 60 weeks. The clinical response of the patients was not uniform and is summarized in Table 3 and 4. Treatment with the non-steroidal anti-rheumatic drugs remained unchanged in three of the patients throughout the study. In one patient (B.K.) the dose had to be increased and in another patient (W.R.) it was reduced and finally discontinued. Blood sedimentation rate monitored as a parameter of inflammation decreased in only one patient (W.R.), from a high value to normal. In all the other patients there were no significant changes.

Four of the five patients reported a reduction of the intensity of pain which amounted to more

TABLE 3. Photopheresis in psoriatic arthritis: clinical results I

Patient	Week of treatment	Grip strength left/right (mm/Hg)	Pain score*	BSR
B.K.	0	300/300	2	16/40
	20	300/300	2	23/50
	32	300/300	2	28/45
K.M.	0	230/035	2	28/155
	20	210/165	1	62/96
S.M.	0	050/050	3	65/85
	20	050/055	2	60/90
	56	120/140	1	50/92
W.R.	0	300/300	2	70/105
	20	250/300	1	28/50
	60	300/300	0	10/20
D.H.	0	140/220	1	13/29
	20	250/300	1	8/25
	34	250/300	0	5/11

* Pain score: 0, absent; 1, slight; 2, moderate; 3, severe.

TABLE 4. Photopheresis in psoriatic arthritis: clinical results I

Patient	Week of treatment	Morning stiffness (min)	Joint swelling (mm)	Skin score
B.K.	0	5	59	17
	20	30	62	17
	32	0	63	17
K.M.	0	60	68	93
	20	60	68	69
S.M.	0	15	62	139
	20	0	62	74
	56	0	62	64
W.R.	0	20	70	203
	20	10	65	126
	60	0	61	146
D.H.	0	15	65	142
	20	0	65	79
	34	0	65	85

than one score in two patients (S.M., W.R.). Morning stiffness was of shorter duration in four patients, whereas a significant change of the grip strength was observed in only two patients (S.M., D.H.). A clear decrease of swelling of indicator joints occurred in one patient (W.R.) who had experienced an acute bout of arthritis immediately after initiation of photopheresis therapy. The girth of the indicator interphalangeal joint increased in one patient (B.K.) but remained unchanged in all other patients.

When the objectively measurable parameters of grip strength and swelling were considered there was a definite improvement in three patients (S.M., W.R., D.H.). Also with the subjective parameters, four of the five patients showed an improvement in more than two parameters. In one patient (S.M.) an improvement of the radiographic changes was seen that could be attributed to therapy. This patient also showed considerable improvement as regards grip strength and pain.

In one patient (B.K.) the condition worsened during treatment and the subjective symptoms became more severe, necessitating an increase in the dosage of NSAR. One patient (W.R.) suffering from severe, recurrent attacks of arthritis showed a definite improvement concerning the subjective symptoms and the dosage of NSAR was reduced and finally discontinued. He is still in remission 1 year after photopheresis was discontinued. The remaining three patients improved but were never completely free of symptoms. The lesions of psoriasis showed only minimal change and additional therapy with anthralin, topical corticosteroid preparations, and/or UVB irradiation was necessary.

In all these patients indicator lesions had been left untreated to permit the evaluation of possible effects of photopheresis on skin lesions. The skin score of the patients and its changes during the course of the treatment is shown in Table 4; no clear improvement of psoriatic skin lesions was observed.

Side-effects

In general, the treatment was well tolerated. Slight symptoms of circulatory dysregulation (short periods of dizziness, hypertension) occurred twice in one of our patients (D.H.) Another (B.K.) complained of a painful light sensitivity of his eyes and this lasted for a few days following each treatment. Ophthalmologic examination did not reveal anything which could have explained this problem. The haematologic and biochemical parameters showed only slight change during therapy.

DISCUSSION

Extracorporeal photochemotherapy induces damage to peripheral white blood cells,[21,22,28] and studies are in progress to determine the effects on inflammatory diseases and on autoimmune processes.[29] It has been suggested that the large number of damaged cells induce immunologically relevant down-regulatory processes after their re-infusion and subsequent uptake of the damaged lymphocytes by the reticuloendothelial system. This hypothesis is based on the assumption that T cells with anti-idiotypic activity are formed and inhibit either autonomously or reactively proliferating T-cell clones.[23] Experiments with animal models[30,31] show that photopheresis affects autoimmune processes, and this may explain the successful treatment of a patient with pemphigus vulgaris.[28]

The present pilot study was intended to evaluate the efficacy of photopheresis in psoriatic arthropathy. Our data confirms that this treatment is safe but long-term follow-up will be necessary. Since extracorporeal photochemotherapy represents an expensive and time-consuming procedure we were unable to perform a controlled double-blind study. Therefore, placebo effects, seasonal variations in disease activity and other factors may have influenced the results. The five patients treated showed a slight to moderate improvement but further, large and controlled studies using photopheresis in inflammatory conditions are required.

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Superior antibacterial action and reduced incidence of bacterial resistance in minocycline compared to tetracycline-treated acne patients

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SUMMARY

Twenty-five previously untreated acne patients were monitored throughout a 6-month course of therapy with either tetracycline or minocycline for changes in the numbers of staphylococci, propionibacteria and yeasts of the genus *Malessezia* on the skin surface. Antibiotic resistant staphylococci and propionibacteria were also counted. Minocycline (50 mg b.d.) produced a 10-fold greater reduction in propionibacterial numbers compared to tetracycline (500 mg b.d.) after 12 ($P < 0{\cdot}02$, *t*-test) and 24 weeks ($P < 0{\cdot}05$) of therapy. As treatment progressed, propionibacteria were replaced by yeasts, numbers of which were significantly increased by week 12 ($P < 0{\cdot}02$) in tetracycline-treated patients and by week 24 ($P < 0{\cdot}01$) in minocycline-treated patients. This suggests that yeasts have no role in the pathogenesis of acne but may compete with propionibacteria for the same niche. Overgrowth of antibiotic resistant staphylococci prevented any decrease in staphylococcal numbers in tetracycline-treated patients, but minocycline produced a significant and sustained reduction in staphylococcal numbers after 1 week of therapy ($P < 0{\cdot}001$). An increase in the number of multiply resistant ($\geqslant 3$ resistances) staphylococci occurred in 67% of tetracycline-treated and 33% of minocycline-treated patients by the end of the treatment period. There was no evidence of propionibacterial resistance in either treatment group. This study shows that minocycline has much greater antibacterial activity *in vivo* against both staphylococci and propionibacteria and produces less staphylococcal antibiotic resistance than tetracycline.

Tetracyclines have been the antibiotics of choice for acne therapy for almost 30 years. Various studies have compared the efficacy of the different tetracyclines currently available for the treatment of acne.[1–6] No clear evidence has emerged to prove the superiority of any of the newer tetracyclines (e.g. doxycycline, minocycline) over the parent compound (tetracycline HCl). However, in the main, comparisons have been made based on clinical evidence alone. The present study was undertaken to compare the anti-microbial efficacy of minocycline and

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tetracycline and the effects of their prolonged administration on the incidence of antibiotic-resistant staphylococci and propionibacteria, and to determine whether one tetracycline was superior to another on microbiological grounds.

METHODS

Subjects

Twenty-five acne patients were studied. None had previously been treated with oral antibiotics for acne nor had they received any antibiotic, other than penicillin, in the 12 months prior to the study. The patients were divided into two groups, one of which (seven females, five males; mean age 17·1 ± 1·54 years) received 500 mg b.d. tetracycline (Achromycin, Lederle, Gosport, Hants, U.K.). The other group (seven females, six males; mean age 16·5 ± 1·39 years) received 50 mg b.d. minocycline (Minocin, Lederle). Treatment was continued for 6 months. No adjunctive therapy was permitted. Patients were assessed pretreatment (two visits) and after 1, 12 and 24 weeks of therapy.

Microbiology

Sample collection. Bacteria and yeasts were obtained from the skin surface (right cheek) by a detergent scrub technique.[7] Viable counts were obtained by plating out serial 10-fold dilutions of the wash fluid onto Brain Heart Infusion agar (incubated anaerobically at 37°C for 6 days) for isolation of propionibacteria, onto heated blood agar (incubated aerobically at 37°C for 2 days) for isolation of staphylococci and onto a novel milk-based medium[8] (incubated at 34°C for 14 days in a moist chamber) for isolation of yeasts of the genus *Malessezia (Pityrosporum)*.

Detection of antibiotic resistance. Antibiotic-resistant propionibacteria were counted directly from skin by plating-out 10-fold dilutions of wash fluid onto Brain Heart Infusion agar containing tetracycline (3·2 or 12·8 mg/l) or minocycline (1·6 or 6·4 mg/l). The medium was supplemented with 2 mg/l of furazolidone to prevent the growth of staphylococci.[9] Staphylococci resistant to a variety of antibiotics were detected by plating-out wash fluid directly onto IsoSensitest agar containing tetracycline (12·8 mg/l), minocycline (6·4 mg/l), erythromycin (5 mg/l), clindamycin (5 mg/l), trimethoprim (20 mg/l), fusidic acid (2 mg/l), chloramphenicol (10 mg/l) or gentamicin (5 mg/l). Each different staphylococcal colony type from the selective and

TABLE 1. Microbial counts before treatment

	Mean $\log_{10}$ cfu*/cm^2 skin ± SEM before treatment	
	Tetracycline-treated patients	Minocycline-treated patients
Total staphylococci	4·23 ± 0·25	4·34 ± 0·26
Tet^R staphylococci	2·10 ± 0·34	2·65 ± 0·47
Min^R staphylococci	0	0
Propionibacteria	4·78 ± 0·25	4·67 ± 0·30
Malessezia spp.	2·47 ± 0·42	2·67 ± 0·30

* cfu, colony forming unit.

non-selective media (a minimum of six isolates per patient) was purified, re-tested for antibiotic resistance and stored. The isolates were then biotyped by the method of Kloos and Schleifer[10] and tested for multiple resistance to 18 antibiotics. Resistances were recorded after 18–24 h incubation at 37°C.[11] Table 1 shows the mean counts of the various microbial groups before the start of therapy.

Clinical assessment

Clinical improvement was assessed on each visit by acne grading.[12]

Statistical analyses

Results were analysed using Student's *t*-test.

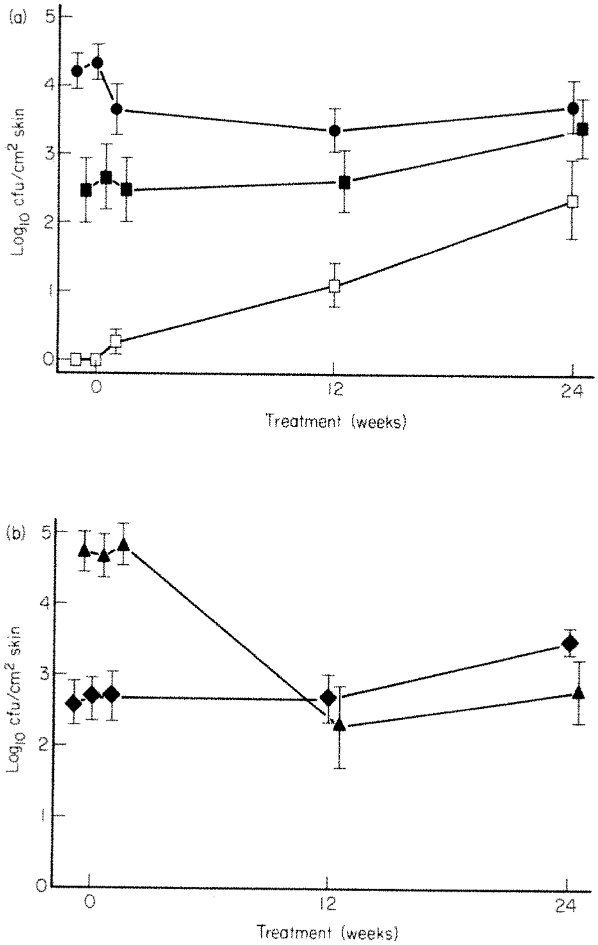

FIGURE 1. Changes in microbial numbers during minocycline therapy. (a) Changes in total staphylococcal numbers (●——●), and in numbers of tetracycline (■——■) and minocycline (□——□) resistant staphylococci. (b) Decrease in propionibacterial numbers (▲——▲) and the increase in yeasts of the genus *Malessezia* (◆——◆).

RESULTS

Effects on microbial numbers

Total and antibiotic-resistant staphylococci. The effects of minocycline and tetracycline on total staphylococcal numbers at the skin surface are shown in Figures 1 and 2 respectively. Minocycline produced a significant and sustained reduction in total staphylococci after 1 week of therapy (Table 2). None of the patients carried any minocycline-resistant (Min^R) staphylococci before treatment. There was a significant rise in the number of Min^R staphylococci after 12 and 24 weeks of minocycline therapy ($P < 0{\cdot}01$). However, there was considerable variation between patients, ranging from a total absence of Min^R strains after 24 weeks to the complete replacement of the original sensitive flora with Min^R staphylococci. Minocycline therapy did not result in a significant increase in tetracycline-resistant (Tet^R) staphylococci. After 24 weeks, six patients carried more Tet^R staphylococci than before treatment, four patients carried fewer and two showed no overall change.

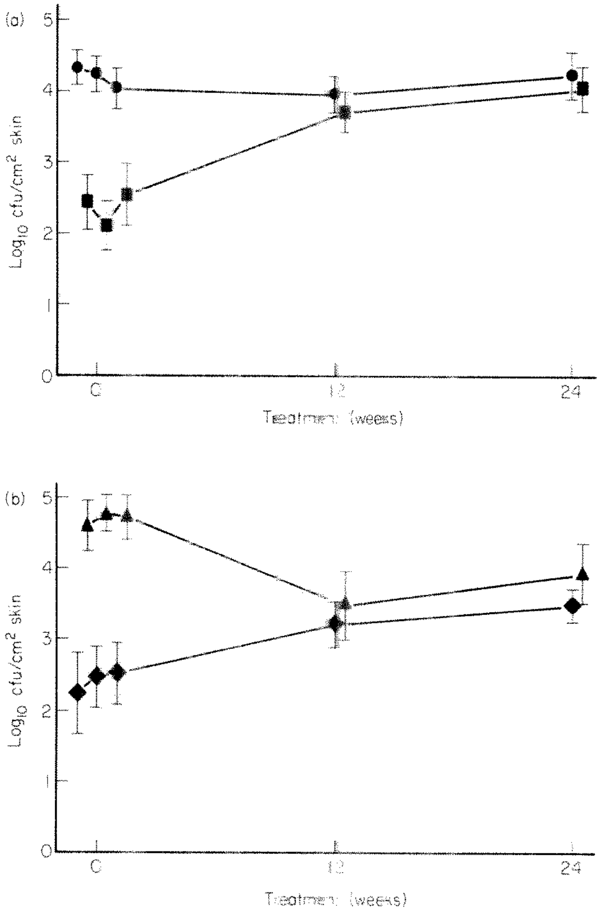

FIGURE 2. Changes in microbial numbers during tetracycline therapy. Legend as for Figure 1.

TABLE 2. Statistical significances of temporal changes in microbial numbers during tetracycline and minocycline therapy

Microbial group	Weeks from start of therapy	Significance (Student's *t*-test) Minocycline	 Tetracycline	 Minocycline vs. Tetracycline
Total staphylococci	1	$\downarrow P<0{\cdot}001$	$P=\mathrm{NS}$	$P=\mathrm{NS}$
	12	$\downarrow P<0{\cdot}02$	$P=\mathrm{NS}$	$P=\mathrm{NS}$
	24	$\downarrow P<0{\cdot}02$	$P=\mathrm{NS}$	$P=\mathrm{NS}$
Tetracycline resistant staphylococci	1	$P=\mathrm{NS}$	$P=\mathrm{NS}$	$P=\mathrm{NS}$
	12	$P=\mathrm{NS}$	$\uparrow P<0{\cdot}001$	$P<0{\cdot}01^{*}$
	24	$P=\mathrm{NS}$	$\uparrow P<0{\cdot}001$	$P=\mathrm{NS}$
Total propionibacteria	1	$P=\mathrm{NS}$	$P=\mathrm{NS}$	$P=\mathrm{NS}$
	12	$\downarrow P<0{\cdot}001$	$\downarrow P<0{\cdot}01$	$P<0{\cdot}02^{\dagger}$
	24	$\downarrow P<0{\cdot}001$	$\downarrow P<0{\cdot}05$	$P<0{\cdot}05^{\dagger}$
Total yeasts of the genus *Malassezia*	1	$P=\mathrm{NS}$	$P=\mathrm{NS}$	$P=\mathrm{NS}$
	12	$P=\mathrm{NS}$	$\uparrow P<0{\cdot}02$	$P<0{\cdot}05^{\ddagger}$
	24	$\uparrow P<0{\cdot}01$	$\uparrow P<0{\cdot}01$	$P=\mathrm{NS}$

↓, Significant decrease from T_0 (untreated).

↑, Significant increase from T_0 (untreated).

NS, Not significant.

*, Tetracycline produced a greater increase in $\mathrm{Tet}^{\mathrm{R}}$ staphylococci than minocycline.

†, Minocycline produced a greater decrease in propionibacterial numbers than tetracycline.

‡, Tetracycline produced a greater increase in numbers of yeasts of the genus *Malassezia* than minocycline.

In contrast, overgrowth of $\mathrm{Tet}^{\mathrm{R}}$ staphylococci prevented any decrease in total staphylococcal numbers in tetracycline-treated patients throughout the treatment period (Fig. 2, Table 2). By week 24 the count of $\mathrm{Tet}^{\mathrm{R}}$ staphylococci approximated to the total staphylococcal count before treatment in all but two tetracycline-treated patients.

One minocycline-treated patient carried $3{\cdot}4\times10^{5}$ more staphylococci/cm^2 skin at the end of the treatment period than at the start. This patient experienced a considerable flare of his acne between weeks 12 and 24, which may have been due to a staphylococcal folliculitis.

Total and antibiotic-resistant propionibacteria. Both tetracycline and minocycline produced a > 1 log-fold mean reduction in propionibacterial numbers by week 12 (Figs 1 and 2). However, minocycline produced a 10-fold greater reduction than tetracycline ($P<0{\cdot}02$, Table 2). Between weeks 12 and 24, numbers of propionibacteria began to rise in both treatment groups but remained significantly depressed compared to pretreatment levels. None of the patients yielded any $\mathrm{Tet}^{\mathrm{R}}$ or $\mathrm{Tet}^{\mathrm{R}}\mathrm{Min}^{\mathrm{R}}$ isolates throughout the treatment period.

Malassezia spp. In minocycline-treated patients the numbers of yeasts remained unaltered during the first 12 weeks of therapy. By week 24 there was a significant increase in the number of yeasts present at the skin surface (Fig. 1, Table 2). In tetracycline-treated patients the rise began earlier so that a significant increase had occurred by week 12 (Fig. 2, Table 2).

Multiply-resistant staphylococci. Six minocycline-treated and seven tetracycline-treated patients carried no multiply-resistant staphylococci at T_0. In the majority of patients there was very little oscillation ($\leqslant 1\%$ change) between the two pretreatment visits. However, three patients who went on to be treated with minocycline showed fluctuations of > 2 log in the number of multiply-resistant staphylococci between T_{-1} and T_0. As treatment progressed the number of tetracycline-treated patients carrying multiply-resistant staphylococci rose steadily (Table 3). There was a similar but less obvious trend in the minocycline-treated patients. In general, minocycline-treated patients who did not develop significant numbers of Min^R staphylococci tended to show either a decrease in the number of multiply-resistant strains or no overall change. In contrast, minocycline-treated patients who did develop Min^R staphylococcal floras tended to show a concomitant increase in multiple resistance. The changes in the carriage of multiply-resistant staphylococci are illustrated for three patients in Table 4. Subjects V.M. (tetracycline-treated) and A.S. (minocycline-treated) showed an increase in multiply-resistant strains, whereas subject A.C. (minocycline-treated) showed a decrease compared with T_0. The staphylococcal strains that were predominant (i.e. present in greater numbers than any other single strain) in each subject at the end of treatment are shown in Table 4. Despite the fact that the majority of tetracycline-treated patients carried multiply-resistant staphylococci, the predominant strain often had a simple resistance pattern—either Tet^R or Tet^RPen^R. This was less likely to be the case in minocycline-treated patients. In five out of six patients in whom the predominant strain was Min^R, it was also multiply-resistant. In only five out of 24 patients was the predominant strain identified as a species other than *S. epidermidis* (Table 5). Neither tetracycline nor minocycline selected for staphylococci-carrying resistances to aminoglycosides (gentamicin, kanamycin, neomycin, tobramycin or amikacin), rifampicin, vancomycin or cephalothin either singly or as part of a multiply-resistant phenotype. One patient acquired

TABLE 3. Temporal changes in the carriage of multiply resistant staphylococci during minocycline and tetracycline therapy

		Number of patients with change in count at T (weeks from start of therapy)			
Antibiotic		−1*	+1	+12	+24†
Minocycline ($n = 13$)	Increase	2	0	4	4
	No change	6	10	6	5
	Decrease	2	3	3	3
Tetracycline ($n = 12$)	Increase	1	3	7	8
	No change	9	7	4	3
	Decrease	1	2	1	1

* Only 10 minocycline and 11 tetracycline-treated patients sampled.

† Only 12 minocycline-treated patients sampled.

Initially, six minocycline- and seven tetracycline-treated patients carried no multiply resistant staphylococci.

TABLE 4. Incidence of multiply-resistant staphylococci in individual patients

Patient	Therapy	Number* and types of multiply-resistant staphylococci			
		T$_0$†	T$_1$	T$_{12}$	T$_{24}$
V.M.	Tetracycline (5·40 × 10^3)‡	None	8·32 × 10^1 *S haemolyticus* Er^{R}Lm^{I}Fus^{R}Chlor^{R}Pen^R	3·65 × 10^2 *S. epidermidis* Tet^{R}Em^{R}Pen^R	1·50 × 10^2 *S. epidermidis* Tet^{R}Fus^{R}Pen^R
				S. epidermidis Tet^{R}Fus^{R}Tmp^{R}Pen^R	*S. epidermidis* Tet^{R}Fus^{R}Tmp^{R}Pen^R
				S. epidermidis Tet^{R}Em^{R}Lm^{R}Fus^{R}Tmp^{R}Pen^R	*S. epidermidis* Tet^{R}Em^{R}Lm^{R}Fus^{R}Tmp^{R}Pen^R
					S. haemolyticus Em^{R}Lm^{R}Fus^{R}Chlor^{R}Pen^R
A.C.	Minocycline (5·40 × 10^4)	2·08 × 10^3 *S. epidermidis* Tet^{R}Em^{R}Fus^{R}Pen^R	None	None	6·16 × 10^2 *S. epidermidis* Tet^{R}Min^{R}Em^{R}Fus^{R}Pen^R
		S. epidermidis Tet^{R}Em^{R}Lm^{I}Tmp^{R}Pen^R			*S. epidermidis* Em^{R}Fus^{R}Pen^R
		S. epidermidis Em^{R}Lm^{I}Tmp^{R}Pen^R			*S. epidermidis* Em^{R}Fus^{R}Pen^{R}Meth^R
A.S.	Minocycline (4·60 × 10^5)	None	None	None	4·58 × 10^4 *S. epidermidis* Tet^{R}Min^{R}Fus^{R}Pen^R
					S. epidermidis Tet^{R}Min^{R}Em^{R}Lm^{R}Fus^{R}Pen^R

* Number of multiply-resistant isolates/cm^2 skin. Note. Tet^{R}Min^R and Em^{R}Lm^R (or Em^{R}Lm^I) each counted as one determinant.

† Weeks from start of therapy.

‡ Total staphylococcal count at T$_0$.

Abbreviations: Chlor, chloramphenicol; Em, erythromycin; Fus, fusidic acid; I, inducibly resistant; Lm, clindamycin; Meth, methicillin; Min, minocycline; Pen, penicillin; R, resistant; Tet, tetracycline; Tmp, trimethoprim.

TABLE 5. Predominant staphylococcal strains at the end of treatment

Predominant strain* and resistance pattern			
Tetracycline-treated patients		Minocycline-treated patients	
R.B.†	*S. hominis* Tet^{R}	T.B.	*S. hominis* Pen^{R}
P.R.	*S. capitis* Tet^{R}	S.M.	*S. epidermidis* Pen^{R}
B.G.	*S. haemolyticus* Tet^{R}	M.T.	*S. epidermidis* Tet^{R}Pen^{R}
V.G.	*S. epidermidis* Tet^{R}Pen^{R}	D.T.	*S. epidermidis* Tet^{R}Pen^{R}
M.S.	*S. epidermidis* Tet^{R} and Tet^{R}Pen^{R}		
S.P.	*S. epidermidis* Tet^{R}Pen^{R}	S.P.	*S. epidermidis* Tet^{R}Pen^{R}
V.M.	*S. epidermidis* Tet^{R} and Tet^{R}Pen^{R}	T.E.	*S. epidermidis* Tet^{R}Pen^{R} and Tet^{R}Min^{R}Pen^{R}
T.F.	*S. epidermidis* Tet^{R}Pen^{R} and Tet^{R}Min^{R}Pen^{R}		
D.W.	*S. epidermidis* Tet^{R} and Tet^{R}Pen^{R}	A.C.	*S. epidermidis* Em^{R}Fus^{R}Pen^{R}
M.E.	*S. epidermidis* Em^{R}Fus^{R}Pen^{R}	A.S.	*S. epidermidis* Tet^{R}Min^{R}Fus^{R}Pen^{R}
D.F.	*S. epidermidis* Tet^{R}Em^{R}Pen^{R}	N.F.	*S. epidermidis* Tet^{R}Min^{R}Fus^{R}Pen^{R}
J.B.	*S. epidermidis* Tet^{R}Em^{R}Lm^{I} and Tet^{R}Em^{R}Lm^{I}Pen^{R}	S.W.	*S. hominis* Tet^{R}Min^{R}Fus^{R}Tmp^{R}Pen^{R}
D.B.	*S. epidermidis* Tet^{R}Fus^{R}Pen^{R}	J.R.	*S. epidermidis* Tet^{R}Min^{R}Em^{R}Lm^{R}Fus^{R}Tmp^{R}Pen^{R}
N.N.	*S. epidermidis* Tet^{R}Fus^{R}Tmp^{R}Pen^{R}	G.R.	*S. epidermidis* Tet^{R}Em^{R}Lm^{R}Fus^{R}Tmp^{R}Pen^{R} and Tet^{R}Min^{R}Em^{R}Lm^{R}Fus^{R}Tmp^{R}Pen^{R}

* The predominant strain (detected by colony morphology on the non-selective medium) represented the isolate present in greater numbers than any other individual isolate at the end of the treatment period. Frequently, however, the combination of other isolates totalled more than the count of the predominant isolate.

† Initials of individual patients.

Abbreviations: see Table 4.

TABLE 6. Clinical improvement during tetracycline and minocycline therapy assessed by acne grading

	Minocycline			Tetracycline		
	T_0	T_{12}	T_{24}	T_0	T_{12}	T_{24}
Mean grade (all patients)	0·82±0·19	0·47±0·08	0·36±0·06	0·43±0·06	0·34±0·05	0·20±0·05
Proportion of patients with reduced grade	—	8/13	10/12	—	7/12	9/12
Mean % reduction in acne grade (responders)	—	61·3±5·0	56·0±7·2	—	40·6±8·6	65·5±8·4

Mean ± SEM, in all cases.

small numbers of aminoglycoside-resistant staphylococci, whereas two patients, who carried such strains before treatment, lost them during minocycline therapy. Low numbers of methicillin-resistant staphylococci were present before and/or during therapy in 48% of patients. Multiply-resistant *S. aureus* were not isolated before or during therapy.

Clinical improvement

As expected, both tetracycline and minocycline produced clinical improvement in the majority of patients (Table 6). Although the minocycline-treated patients had a higher mean grade before treatment, the percentage improvement was similar in both groups. Two minocycline and one tetracycline-treated patient showed a flare in their acne at week 24. Gram-negative folliculitis was excluded in all three patients. Staphylococcal overgrowth was the most likely explanation for the flare in one patient. The microbiology of the remaining two patients was normal.

DISCUSSION

This is the first study to simultaneously monitor change in numbers of propionibacteria, staphylococci and yeasts of the genus *Malessezia*, and the development of bacterial resistance, during a standard 6-month course of oral antibiotic therapy for acne vulgaris. The main entry criteria, that the patients were previously untreated, minimized the risk that the microflora was already perturbed by earlier therapy, particularly antibiotic resistance of staphylococci. The observed changes were likely to be simpler than in patients on repeated courses of therapy.

Minocycline at one-half full therapeutic dose produced a 10-fold greater reduction in propionibacterial numbers than tetracycline at full therapeutic doses. A similar superiority of minocycline was found by Leyden, McGinley and Kligman[5] using full therapeutic doses of both antibiotics. Irrespective of the antibiotic used, suppression of propionibacterial numbers was greater after 12 than 24 weeks of therapy. A rising bacterial count during antibiotic therapy usually indicates the development of resistance. However, in all patients the propionibacterial flora remained fully sensitive to both antibiotics. An alternative explanation for the rising propionibacterial count is that, as the acne improved, patients' compliance diminished, so that the antibiotic concentration on skin dipped below a minimum inhibitory level.

As treatment progressed, propionibacteria were replaced by *Malessezia* yeasts in both treatment groups. This supports the view that yeasts have no role to play in the pathogenesis of acne but may compete with propionibacteria for the same niche. One patient showed a 4-$\log_{10}$ increase in yeasts during tetracycline therapy and yet experienced no exacerbation of her acne. Yeast overgrowth is a common complication of the administration of broad-spectrum antibacterial antibiotics and many reports exist of *Candida* overgrowth following oral tetracycline therapy.[13-15] This is the first report of *Malessezia* overgrowth in patients treated with tetracyclines. The recent development of a reliable culture medium for these organisms[8] has made it possible to accurately follow changes in their numbers during treatment. *Malessezia* yeasts have recently been found to cause an increased incidence of seborrhoeic dermatitis and pityriasis versicolor in immunocompromised hosts such as AIDS patients.[16,17] Therefore, the expansion of the cutaneous yeast population may be related to the immunosuppressive activity of the tetracyclines.[18-20] Whatever the cause, the increased number of cutaneous yeasts does not lead to a clinical deterioration in the acne patients.

Minocycline produced a significant and sustained reduction in staphylococcal numbers after 1 week of therapy. Overgrowth of Tet^{R} staphylococci prevented any similar decrease in tetracycline-treated patients. These results support the findings of Marples and Kligman[21] who found that, after 3 weeks of oral therapy, minocycline but not tetracycline produced a significant reduction in staphylococcal numbers on the forehead. Before therapy was initiated 23 out of 25 patients (92%) carried Tet^{R} strains as a proportion of their staphylococcal flora (ranging from 4×10^{0}–8×10^{4} organisms/cm^{2} skin and from 0·025–94% of the total staphylococcal count). None of the patients carried staphylococci expressing Min^{R} before the start of treatment. In staphylococci, resistance to tetracycline is encoded by either plasmid or chromosomally located Tet^{R} genes.[22,23] Tet^{R} genes with a plasmid locus confer resistance to tetracycline but not minocycline. Only chromosomally located Tet^{R} genes confer resistance to minocycline which can be either inducible or, more commonly, constitutive.[22,23] Overgrowth of tetracycline-resistant organisms in tetracycline-treated patients occurs so quickly because of the high level of tetracycline resistance existing before treatment. In contrast, minocycline can inhibit Tet^{R} staphylococci as seen by their failure to proliferate in minocycline-treated patients between weeks 1–12 of treatment. Eventually, in the presence of sustained selective pressure, some staphylococci acquired Min^{R}, but the precise mechanism by which this occurred is unknown. Between weeks 12 and 24 the total number of staphylococci began to rise due to the multiplication of Min^{R} strains. However, staphylococcal numbers were still significantly depressed at the end of the treatment period in minocycline-treated patients.

Two previous studies have reported decreases in staphylococcal numbers with oral tetracycline therapy. Goltz and Kjartansson[24] found a highly significant reduction after 14 days therapy with tetracycline HCl at a dose of 250 mg b.d., the reduction being maintained with a reduced dose of 250 mg once daily for a further 4 weeks. Marples and Williamson[25] also found a reduction in staphylococcal numbers after 8 days therapy with 600 mg demethylchlortetracycline. However, numbers of staphylococci began to rise immediately thereafter as a result of the multiplication of Tet^{R} strains. The reason why our study failed to show a reduction in staphylococcal numbers with oral tetracycline might be explained by the presence of higher numbers of Tet^{R} strains on the skin of untreated subjects than there were 20 years ago.

Multiply-resistant coagulase-negative staphylococci, especially *S. epidermidis*, are becoming increasingly implicated in infections of compromised hosts such as patients on CAPD, premature neonates and those who have undergone cardiac surgery.[26,27] It is important to determine whether antibiotic therapy for acne increases the number of multiply-resistant staphylococci at the skin surface, from where they can be easily disseminated. The enumeration of multiply-resistant staphylococci is a painstaking and time consuming procedure and has not previously been carried out in acne patients treated with oral tetracyclines. Before treatment, 28% of patients carried >100 multiply resistant strains/cm^2 skin (range $1{\cdot}9 \times 10^2$–$1{\cdot}9 \times 10^5$ organisms/cm^2) despite not having received any antibiotic therapy in the 12 months prior to sampling. Tetracycline produced an increase in the number of multiply-resistant strains in 67% of patients by the end of the treatment period. In the case of minocycline, only those patients who developed a Min^R staphylococcal flora showed a concomitant rise in multiple resistance. Although minocycline selected for less multiple resistance overall, resistance patterns of predominant strains tended to be more complex in minocycline-treated patients. Neither antibiotic selected for resistance to the major anti-staphylococcal antibiotics or for antibiotic resistant *S. aureus* at the skin surface.

This study has shown that, during a standard 6-month course of treatment without concomitant topical therapy, minocycline is superior to tetracycline in two important aspects. It demonstrates greater antibacterial activity *in vivo* against both staphylococci and propionibacteria and selects for less staphylococcal antibiotic resistance.

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Mutilating palmoplantar keratoderma with periorificial keratotic plaques (Olmsted's syndrome)

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SUMMARY

We report the case of a boy, now aged 4 years, with a progressive, painful and disabling palmoplantar keratoderma, associated with well-defined hyperkeratotic plaques around the mouth and nostrils. His mother has an identical palmoplantar keratoderma. The palmoplantar keratoderma failed to improve with etretinate, but considerable benefit resulted from full-thickness excision of the skin of both palms, followed by skin grafting. We believe that he has a distinctive but extremely rare form of hereditary palmoplantar keratoderma, first described by Olmsted.

CASE REPORT

A 3-year-old boy presented with palmoplantar keratoderma which started on his soles at the age of 16 months, and later appeared on the palms at the age of 23 months. The keratoderma had initially been focal (Fig. 1a, b), but had extended and thickened rapidly during the 4 months prior to presentation at this hospital. The affected areas were often very painful. His mother regularly shaved down the keratin with a surgical blade, which helped to maintain the function of his hands and feet, and reduced the pain.

He was born at term weighing 2·93 kg (10th centile), and was breast-fed for 3 months. There were no neonatal problems, and he had no cutaneous abnormality other than 'cradle cap' until the age of 16 months.

His parents were unmarried and the boy had no siblings. His father, a West Indian Black, was unrelated to his mother, who was a Jordanian by birth and aged 32 years at the time of her son's presentation. She had less severe palmoplantar keratoderma but it had resulted in the loss of six of her toes and significant disability. She had been abandoned and placed in care in the U.K. in her teens, and at the age of 11 years she was the subject of a published case report.[1] Subsequently, in a series of operations, the skin of her palms and soles was excised down to the subcutaneous aponeurosis, with subsequent split-skin grafting. She still regularly shaves down

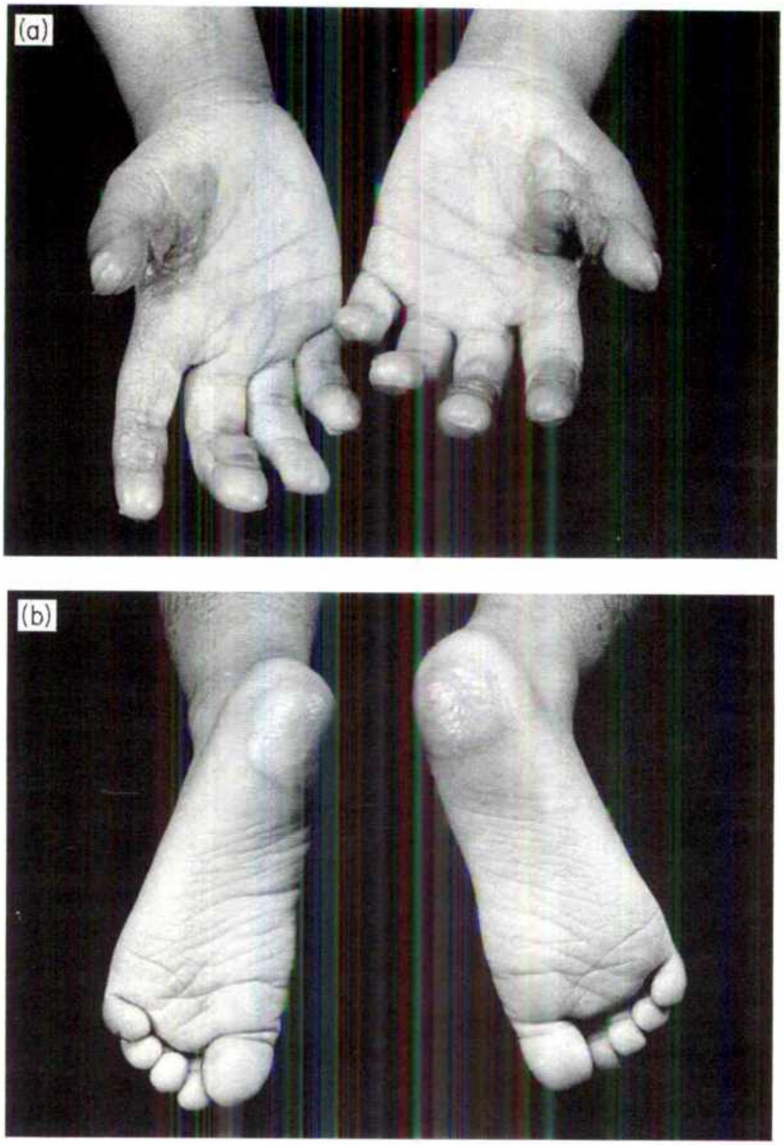

FIGURE 1. (a) Palms and (b) soles at 25 months, showing well-circumscribed, symmetrical areas of focal keratoderma.

remaining hyperkeratotic areas herself. She does not have skin or mucosal lesions other than the palmoplantar keratoderma, and there is no extension beyond the wrist and ankle. She feels great resentment towards her parents, from whom she believes she has inherited the condition, though they were neither related nor affected.

On examination at presentation, our patient's height was 88 cm (10th centile), and his weight was 10·24 kg (below 3rd centile). General examination was unremarkable. His social development was grossly delayed. The entire surface of his palms, soles, and the corresponding

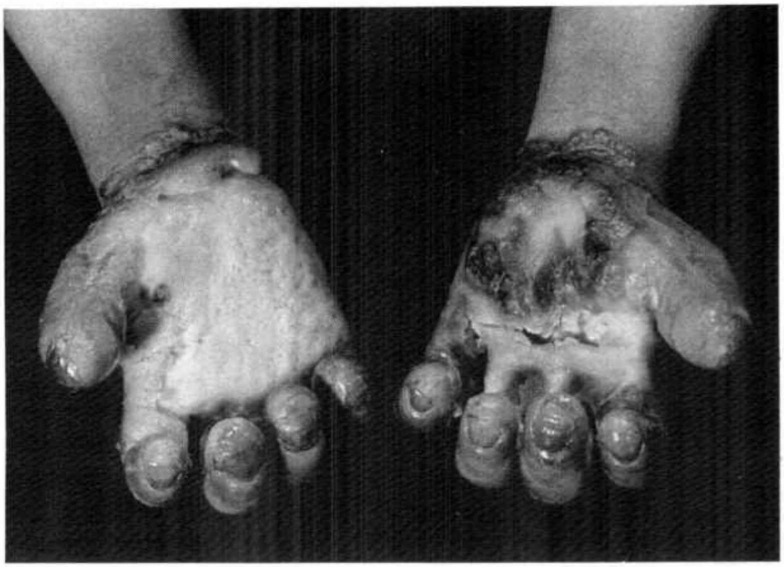

FIGURE 2. Palms at presentation at 40 months, showing diffuse, thick keratoderma, with maceration, fissuring and superficial pitting.

aspects of the fingers and toes showed marked keratoderma, of yellowish colour, macerated texture and with superficial pitting (Fig. 2). The keratoderma extended on to the flexor aspects of the wrists, and on to the lateral aspects of the hands and fingers. Similarly, on the feet, the keratoderma extended slightly on to the lateral aspects of the feet and toes. The borders were erythematous, hyperpigmented and tender, with the keratoderma breaking up into thick flakes. The finger- and toe-nails were all thickened and transversely ridged. The finger joints showed flexion contractures, and there were deep and painful fissures in many of the joint creases of both the fingers and toes. Pus was draining through a defect in the keratin in the centre of the left palm. There was a brownish yellow crusted plaque immediately below the whole length of the lower lip. Otherwise his skin was unremarkable and there was no involvement of the perianal area.

All investigations were normal, other than a delayed bone age of 2 years. His failure to thrive was considered to be due to an emotional cause and his delayed social development was believed to reflect both his emotional deprivation and his failure to become mobile or to develop manual skills because of the painful keratoderma.

His initial treatment comprised 1:8000 potassium permanganate soaks twice daily, with regular application of 10% lactic acid lotion, and a mixture in equal parts of liquid paraffin BP and white soft paraffin BP. He was also given etretinate in a dose approaching 1 mg/kg per day. He failed to gain weight and 3 months after presentation, he was commenced on supplementary overnight nasogastric feeds. Despite these treatments, and the fact that his mother would secretly shave down the thick keratin, the keratoderma became progressively thicker, more warty and fissured (Fig. 3a, b). The palms and soles became extremely malodorous. The survival of his fingers and, particularly, his toes, appeared to be threatened both by the constricting effect of the keratoderma and by the extremely deep fissures that had developed in the flexor creases at their bases. The fingers were becoming increasing flexed, particularly at the

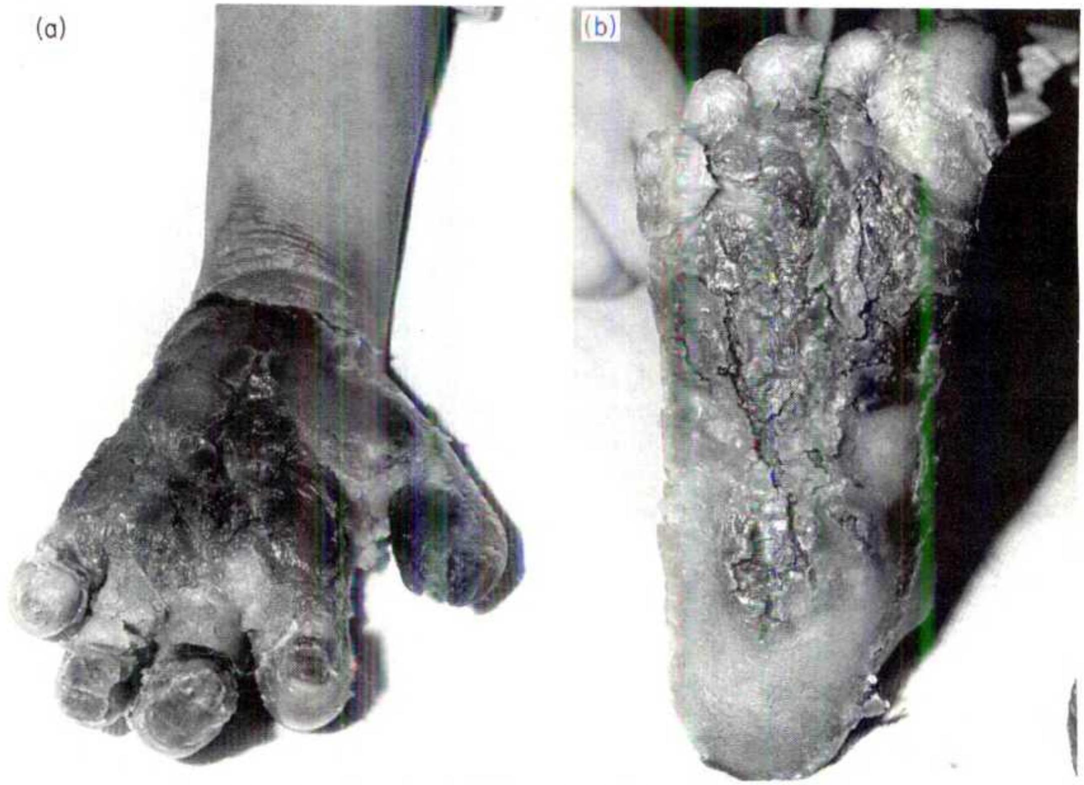

FIGURE 3. (a) Left palm and (b) right sole at the age of 51 months.

proximal interphalangeal joints. The etretinate was discontinued, and we decided to shave down the stratum corneum on the palms and soles at 3–4 weekly intervals, under a general anaesthetic.

Five months after presentation, it was decided to excise the volar skin. The right hand was explored first, under general anaesthetic. It was clear that the joint contractures reflected tension exerted on the phalanges via Cleland's ligaments, due to skin shortening. When the skin was removed all the finger joints exhibited a full range of movement. A split thickness skin graft from the thigh was used to surface the palm, and full thickness inguinal grafts were used for the fingers. Longitudinal Kirschner wires maintained the interphalangeal joints in extension for 6 weeks, following which active physiotherapy was pursued. Two months later, despite physiotherapy and splintage, flexion contractures recurred at the proximal interphalangeal joints. These were released under anaesthesia; further split thickness skin grafts were applied and Kirschner wires inserted. Six weeks later, physiotherapy was re-instituted after the wires had been removed, and the hand was splinted in extension when not in use.

Five months after the original procedure on the right hand, the left was explored. The skin was softer, with no real plane existing between the dermis and the palmar fascia. Consequently, excision was to the deep dermal level only, again over the whole of the palmar and volar digital skin, with a split thickness skin graft to cover the defect. No internal splintage was used, extension being maintained with a Silastic foam dressing. There was a full take of skin graft with no recurrent contracture. Some extension of the keratoderma has occurred just beyond the borders of the grafted areas. We have continued to shave down the plantar keratin every few

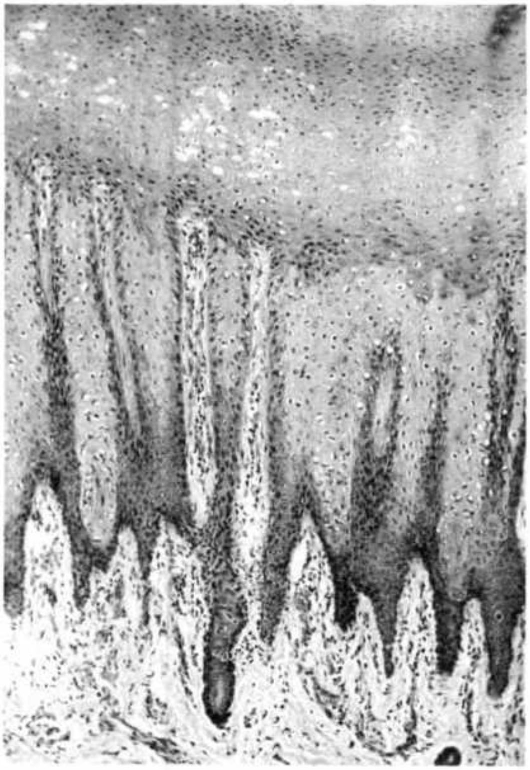

FIGURE 4. Massive hyperkeratosis and parakeratosis of the excised palmar skin. The malpighian layer is greatly thickened and pallid (H&E × 120).

weeks, under general anaesthesia. It is not planned to graft the soles as we fear that the functional result would be poor.

Interestingly, the skin graft donor sites on his anterior thighs became covered by yellowish-brown hyperkeratotic plaques very similar to the perioral lesions, while they were healing. Initially, this seemed to delay healing but was followed by full, normal healing.

Histological examination of the excised palmar skin showed massive thickening of the horny layer in which the nuclei were retained (Fig. 4). On the most superficial surface of the biopsy, the pattern was that of conventional parakeratosis, but lower down in the stratum corneum the nuclei are rounded. The Malpighian layer itself is also markedly thickened, and the keratinocytes, for most of the thickness of the true epidermis, are large and have a slightly pale, hyalinized appearance. In the underlying dermis there is mild inflammation and prominent, rather dilated blood vessels.

In addition to the progression of the palmoplantar keratoderma, the perioral eruption became more extensive and hyperkeratotic. At the time of the grafting of his left hand, there was a hyperkeratotic plaque about 2 cm in width, running below the entire length of the lower lip and including the angles of the mouth, but sparing the vermilion. This plaque had an erythematous border, and closely resembled the skin changes found on the palms and soles. At the age of 51 months, the plaque had extended considerably and a similar lesion ran just below the nose (Fig. 5).

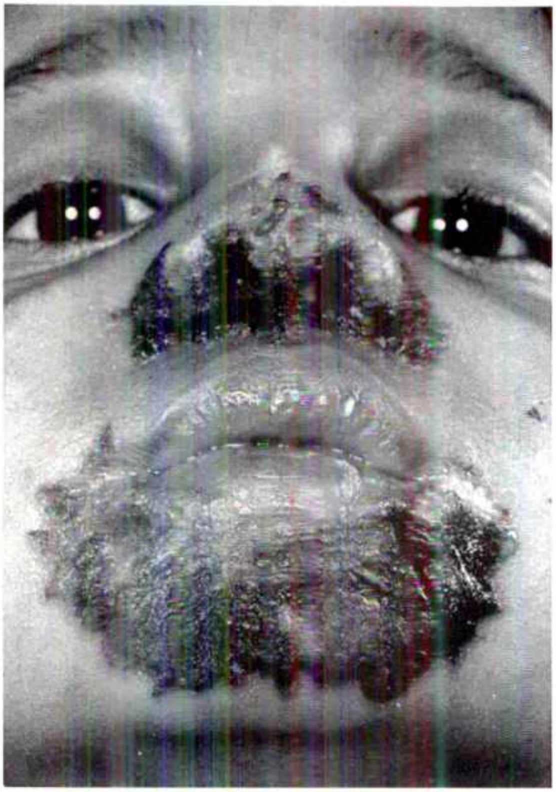

FIGURE 5. Appearance of the mouth and nose at the age of 51 months.

The relationship with his mother did not improve, and he was subsequently taken into the care of the local authority, eventually being discharged to foster parents, in whose care his feeding, weight, mobility and social development have progressively improved.

DISCUSSION

Several cases, both sporadic and familial, have been described in which palmoplantar keratoderma has been complicated by the development of constricting bands of keratin around the bases of digits, eventually leading to their strangulation and autoamputation. The great majority of these cases have demonstrated other clinical features that have helped to identify a distinctive type of keratoderma, now generally known either as 'mutilating keratoderma', or as 'Vohwinkel's syndrome'.[2] In addition to the tendency to autoamputation of digits, which generally becomes apparent during the second decade of life, the characteristic components of this condition include palmoplantar keratoderma, with a 'honeycombed' surface, 'knuckle-pads' and/or 'starfish'-shaped keratoses of the dorsa of the hands and feet, and linear keratoses of the elbows and knees.[3,4]

Constriction and spontaneous amputation of the digits has also been reported in three individuals who appear to have had a distinctive syndrome first described by Olmsted.[5] This case was a 5-year-old boy with thick, deeply fissured palmoplantar keratoderma which had appeared during the second 6 months of life, and which led to spontaneous amputation of the terminal phalanges of two of his fingers.[5] The palmoplantar keratoderma was accompanied by a facial eruption, described as comprising a 'row of yellow-brown plaques of epidermis, interrupted by fissures, and situated parallel to the mucocutaneous junction of the lower lip and the angles of the mouth', and a rather similar eruption in the perianal area. The clinical photographs of the palmoplantar keratoderma and the facial eruption show a striking resemblance to the lesions seen in our patient.

A very similar case was later illustrated by Costa under the title 'polykeratosis of Touraine: pluriorificial keratosis of Olmsted',[6] and two other similar cases were subsequently reported by Ruiz-Maldonado and Lozano-Ferral,[7] and by Poulin and colleagues.[8] These cases show striking resemblances to our own patient. In one,[8] psychomotor development is noted to have been normal at the age of 2 years, and the patient is said to have been of 'normal mental habitus', but of short stature at the age of 21 years. In this patient, the perianal area, the groins and inner thighs, the perioral and perinasal areas, the ears and the anterior neck, were all recorded as involved by the age of 2 years. This patient also featured total alopecia and joint hypermobility, and at the age of 3 years, a linear hyperkeratotic streak had started to extend up both wrists from the palms. By the age of 21 years, several of these streaks had developed, extending from the palms up the flexor forearms, and linear keratoses had appeared in the antecubital fossae. One of the main differences between our own and the four previously reported cases is the absence of perianal involvement in our patient, though this may still develop. We have the impression that the cutaneous plaques may, to some extent, develop as a response to trauma, as in our child's skin graft donor site, so that the distribution would be likely to vary somewhat between cases. Another difference is the absence of oral leukokeratosis, which was present in two of the four previously published cases.[6,8]

Despite the minor differences, we believe that our patient has the same condition described in these four previous cases, i.e. Olmsted's syndrome. Whereas the previous cases had occurred sporadically, in ours there is clear transmission from mother to son and it is likely that the condition behaves genetically as an autosomal dominant trait.

Although the longer-term value of the grafting procedures remains to be established, the result in the short-term has been gratifying, and it is hoped that there will be a decreased risk of spontaneous digital amputation. Certainly, the similar operations undertaken on his mother have provided long-term benefit, and she has since lost no further digits. We anticipate, however, that the keratoderma will extend beyond the grafts at their periphery, and that further surgery may be necessary.

It is difficult to decide whether his retarded growth and psychomotor development were direct reflections of his disease, or of his unsatisfactory social circumstances. This remains unclear, although it is interesting that Olmsted's own case was reported to have somewhat delayed motor and speech development, and was described as having a poor appetite, being malnourished and of small size. The case of Poulin *et al.*[8] was also of short stature but with normal psychomotor development. Perhaps poor growth is a feature of the disease, whereas psychomotor delay is not. The profound and painful palmoplantar keratoderma would interfere with the child's ability and willingness to walk and to develop manual skills.

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Homozygous variegate porphyria: a case report

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SUMMARY

Homozygous variegate porphyria is described in a 14-year-old girl with a unique clinical presentation of photosensitivity from the second year of life, mental retardation, clinodactyly, and normal growth rate. The erythrocyte protoporphyrin concentration was raised with the protoporphyrin being predominantly zinc-chelated, which appears to be characteristic for all homozygous hepatic porphyrias. Protoporphyrinogen oxidase activity in lymphoblasts was decreased in both patient and parents despite the latter having normal porphyrin excretion.

CASE REPORT

W.L., a 14-year-old girl had, from the age of 18 months, recurrent small blisters, crusts and erosions on the sun-exposed areas of skin, especially on the face and backs of hands. They predominantly occurred during the summer. Increased skin fragility of the sun-exposed areas was noticed from the age of five and she had mild flexural atopic eczema from the age of six.

She was born by Caesarian section at 40 weeks gestation and suffered two convulsions of uncertain cause during her first 12 months, but there were no other neuropsychiatric episodes. She was mentally subnormal and attended a special school. Her parents were unrelated and there was no family history suggestive of porphyria.

Examination showed she was of normal height (155 cm) and weight (54·4 kg). The skin of face, backs of hands and knees was dry with crusts, erosions, milia and multiple superficial scars, but no obvious hypertrichosis or sclerosis (Fig. 1). Perioral radial linear scarring was present, as was clinodactyly and brachydactyly of the fifth fingers and flexion deformities of the other fingers. Ophthalmological and neurological examination was normal except for the mental retardation.

Normal or negative investigations were haemoglobin concentration; white cell count total and differential and platelet count; ESR; serum iron concentration and total iron-binding capacity; blood sugar; serum urea and electrolyte concentration; liver function tests; anti-nuclear factor titre; bone age on hand X-ray.

Table 1 shows blood, urine and stool porphyrin concentrations for the patient and her parents. The patient's stool porphyrin concentrations were raised as in variegate porphyria, protoporphyrin being higher than coproporphyrin with the ether insoluble X fraction also elevated. The urinary porphyrin profile was also compatible with variegate porphyria. Fluorescence-emission spectroscopy[1] of plasma from the patient showed a large porphyrin peak

TABLE 1. Laboratory investigations

	Proband	Father	Mother	Normal range
Urine				
Uroporphyrin (μg/l)	145			0–18
Coproporphyrin (μg/l)	184			0–149
Stool				
Coproporphyrin (μg/g dry wt)	131	8	13	0–30
Protoporphyrin (μg/g dry wt)	171	12	17	0–125
Ether insoluble (X) porphyrin (μg/g dry wt)	332	5	5	0–20
Erythrocytes				
Total porphyrin (μg/dl RBC)	645	74	121	0–95
Lymphoblasts				
PO enzyme activity (nmol protoporphyrin/h per mg protein)	0·06	0·28	0·29	0·38–0·47
Ferrochelatase activity (%)	92	69	82	43–92

at 624 nm, from the mother a very small peak at 625 nm and from the father no abnormality. The patient's erythrocyte protoporphyrin was about 80% zinc bound and 20% free protoporphyrin.

Protoporphyrinogen oxidase (PO) measured in Epstein–Barr virus-transformed lymphoblast lines as described by Brenner and Bloomer[2] using protoporphyrinogen purified by reverse-phase chromatography[3] was markedly reduced in the patient and marginally reduced in both parents (Table 1).

Ferrochelatase activity was estimated in mitogen-stimulated lymphocytes by measuring the rate of formation of protoporphyrin from 5-aminolaevulinate in the presence and absence of iron[4] using desferrioxamine to ensure iron deficiency.[5] The difference between rates of protoporphyrin formation with and without iron, expressed as a percentage of the rate without iron, is proportional to ferrochelatase activity. Using this technique ferrochelatase activity was normal in lymphocytes from both proband and her parents (Table 1).

In view of the abnormal stool and urinary porphyrin excretion and erythrocyte porphyrin values, the reduced PO activity and the characteristic fluorescence spectroscopy, the patient was diagnosed as having homozygous variegate porphyria. The parents' PO activity was just below the normal lower limit and therefore compatible with heterozygous porphyria. The marginal elevation of the mother's erythrocyte porphyrin concentration was thought to be secondary to mild coexistent anaemia.

DISCUSSION

In variegate porphyria (VP), an enzyme defect in the haem biosynthetic pathway occurs at the protoporphyrinogen oxidase (PO) step, the activity of this enzyme being reduced by approximately 50%.[6] This defect is evidently inherited as an autosomal dominant trait. Subjects with the trait generally remain asymptomatic throughout life, apart from a minority who develop clinical manifestations, often precipitated by exogenous factors, at some time after

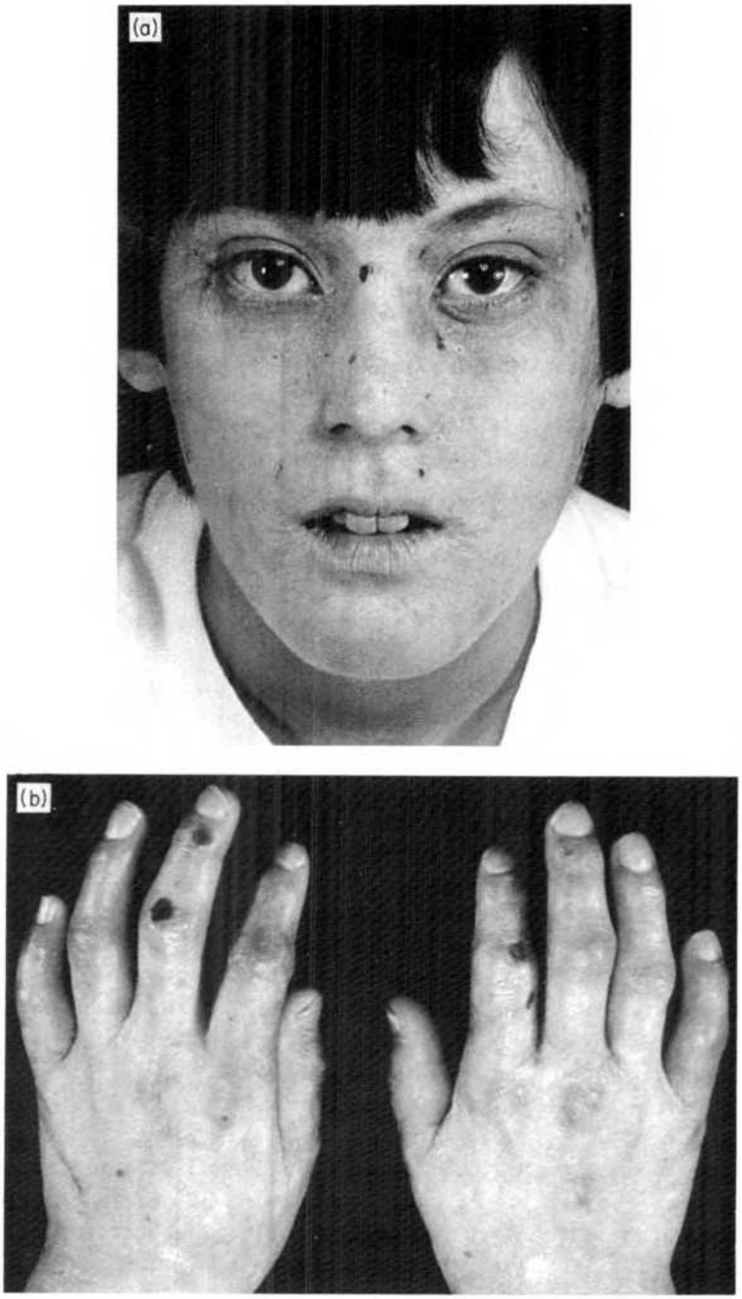

FIGURE 1. Skin of (a) face and (b) backs of hands showing superficial scarring and erosions.

TABLE 2. Details of reported cases of homozygous variegate porphyria

Report	No. of cases	Age onset (months)	Clinical features Photo-dermatosis	Mental retardation	Convulsions	Clinodactyly	Growth retardation
Kordac *et al.*[8]	2	< 1	+	+	+	–	–
Murphy *et al.*[9]	2	< 24	+	–	?	+	+
Mustajoki *et al.*[10]	1	< 1	+	–	–	+	+
Proband (WL)		18	+	+	+	+	–

puberty.[7] The main clinical features are a photosensitized increase in skin fragility, blistering, milia formation and hypertrichosis of the sun-exposed areas, with intermittent sometimes life-threatening acute episodes of abdominal, neurological and psychiatric disorders. In our patient, the characteristic porphyrin excretory abnormalities of VP are associated with raised erythrocyte protoporphyrin level and very low PO activity (less than 20% of normal) while both parents have PO activities close to half-normal. These findings are those of the homozygous form of VP.

Homozygous VP was first described by Kordac *et al.*[8] in two Czech siblings, both with marked photosensitivity in the first days of life, mental retardation, convulsions and nystagmus. Three cases reported subsequently[9,10] have had onset in later childhood with similar cutaneous manifestations, without mental retardation or major central nervous system abnormalities, and have in addition demonstrated growth retardation and clinodactyly (Table 2). Existence of two separate subgroups of homozygous VP appears unlikely however, since our patient has both central nervous system involvement and clinodactyly without growth retardation. Thus, a cluster of clinical manifestations of homozygous VP variably expressed and unrelated to age of onset seems most likely.

Acute abdominal or neuropsychiatric episodes are expected in VP, and their absence in our patient and the others so far reported is interesting since the very low PO activity might be expected to result in increased susceptibility to this complication. Thus, other factors seem likely also to be important in leading to this problem.

Our finding of raised erythrocyte protoporphyrin, predominantly zinc-chelated, has been reported previously in homozygous variegate porphyria.[9-11] This accumulation of protoporphyrin is also present in hepatoerythropoietic porphyria (homozygous porphyria cutanea tarda)[12] and in homozygous hereditary coproporphyria[13] and appears to be a general consequence of severe haem biosynthetic enzyme defects. It may be a useful diagnostic marker for all homozygous hepatic porphyrias.

Both parents of our patient were asymptomatic with normal faecal porphyrin concentrations, despite the presence of the gene for VP suggested by their half-normal PO activity. Similarly, apart from the maternal relatives of one patient,[9] all homozygous VP patients' parents have also been reported as asymptomatic. While this might suggest different defects of the PO enzyme in homozygous and classical VP, Mustajoki[7] has shown that complete latency in VP is not unusual, occuring in over half of affected Finnish patients. However, the possibility cannot yet be excluded that VP, like acute intermittent porphyria[14] and familial porphyria cutanea tarda,[15] is genetically heterogeneous.

Further studies are needed to characterize the exact defect in homozygous VP. As more patients continue to present, particularly from families with atypical findings, the typical nature of the condition will probably emerge.

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PAPERS

Isotretinoin treatment of severe acne affects the endogenous concentration of vitamin A in sebaceous glands. A.VAHLQUIST, O.ROLLMAN,* D.HOLLAND† AND W.J.CUNLIFFE,† *Department of Dermatology, Universities of Linköping and *Uppsala, Sweden, and †Department of Dermatology, The General Infirmary at Leeds, U.K.*

Isotretinoin is a first line drug for systemic treatment of nodulocystic acne although its molecular mechanisms of action are poorly understood. However, since isotretinoin is closely related chemically to the naturally occurring retinoids (retinol, retinoic acid), it is possible that the drug may interfere with the endogenous metabolism of vitamin A. To study the possible interaction of isotretinoin and vitamin A at the target level we investigated 22 patients (aged 15–41 years) with severe nodulocystic acne. Drug and vitamin A levels were determined by HPLC in extracts of micro-dissected sebaceous glands obtained before and during therapy with 1 mg/kg per day of oral isotretinoin. After 2–4 months of treatment the isotretinoin concentrations attained in the sebaceous glands (280–440 ng/g dry weight) were 3–4 times higher than those in the surrounding dermis but somewhat lower than those in the overlying epidermis. The vitamin A content of the sebaceous glands were markedly affected by the treatment. The concentrations of retinol, the predominant form of endogenous vitamin A in the skin, increased 3–5 times as compared to pretreatment levels. In contrast, the concentrations of 3-dehydroretinol, a local metabolite of retinol, decreased to almost undetectable levels. Similar, but less pronounced, changes in the vitamin A composition were found in the epidermis.

The isotretinoin-induced alterations observed suggest that the drug interacts with endogenous vitamin A metabolism in the target tissue. Since 3-dehydroretinol is known to accumulate in hyperproliferative and hyperkeratinizing skin disorders, its reduction following isotretinoin therapy may relate to the inhibition of cellular differentiation or proliferation induced by the drug.

Retinoid metabolism by isolated human sebaceous glands. J.A.E.LATHAM, C.P.F.REDFERN AND A.J.THODY, *Dermatology Department, University of Newcastle upon Tyne, U.K.*

Synthetic retinoids are clinically effective in disorders involving abnormal epithelial cell differentiation. One analogue, 13-*cis* retinoic acid (RA), is particularly effective in the treatment of acne where it causes a reduction in sebaceous gland size and sebum excretion rate, with subsequent clinical improvement and a long-lasting remission from disease. Surprisingly, all-*trans* RA acid does not have these effects on sebaceous gland function although it is a potent modulator of differentiation in other epithelial cells. The different effects of 13-*cis* and all-*trans* RA on sebaceous gland function could be due to differences in their rates of metabolism and stability in this tissue. We have therefore investigated the rates of metabolism of these two stereo-isomers by isolated sebaceous glands *in vitro*.

Human sebaceous glands were isolated and incubated in DMEM with 10% fetal calf serum containing 10^{-7} M all-*trans*-[11,12-^{3}H] or 13-*cis*-[11,12-^{3}H] RA for 1–2 days. Aliquots (100 μl) of the culture medium and of a control culture containing medium but no glands were taken at various time points and analysed by HPLC.

Both isomers were taken up and metabolized by the glands. 13-*cis* RA was metabolized more slowly than all-*trans* RA and the half-life was 48·4 h compared to 16·6 h for all-*trans* RA. Analyses of sebaceous gland lysates suggested that there was interconversion of the stereo-isomers, presumably by an endogenous isomerase. In both cases, polar metabolites were secreted into the medium.

Thus, the slower metabolism of 13-*cis* RA in the sebaceous glands, compared to that of all-*trans* RA, may in part explain the specific therapeutic effect of 13-*cis* RA in acne.

Effect of low-fat diet on retinoid hyperlipidaemia. J.R.MARSDEN, S.SCOTT,* R.L.HOLDER† AND N.COWLEY, *Departments of Dermatology and *Dietetics, University Hospital, Nottingham, and †Mathematics and Statistics, University of Birmingham, U.K.*

It is unclear if reducing dietary fat intake decreases retinoid hyperlipidaemia. Thirty-seven patients (24 male and 13 female, age range 17–41) were treated with isotretinoin 1 mg/kg per day for 20 weeks for severe acne. Group 1 ($n = 13$) had their normal diet for the first 10 weeks of treatment (phase i), and in the second 10 weeks (phase ii) had a low-fat isocaloric diet with no more than 30% total calories from fat. Group 2 ($n = 12$) had a low-fat isocaloric diet in phase i and reverted to normal diet in phase ii, and group 3 ($n = 12$) had a normal diet throughout treatment. Levels of cholesterol (C), triglyceride (T), and high density lipoprotein cholesterol (HDLc) were measured at 2-week intervals from 0–24 weeks. In phase i, C increased by 0·32 mmol/l ± 0·08 SEM in group 1; by 0·30 mmol/l ± 0·10 in group 2 and by 0·49 mmol/l ± 0·10 in group 3, and in phase ii by 0·10 mmol/l ± 0·10 in group 1, by 0·72 mmol/l ± 0·10 in group 2 and by 0·43 mmol/l ± 0·13 in group 3. In phase i, T increased by 0·27 mmol/l ± 0·05 in group 1, by 0·52 mmol/l ± 0·07 in group 2 and by 0·41 mmol/l ± 0·07 in group 3, and in phase ii by 0·36 mmol/l ± 0·08 in group 1, by 0·95 mmol/l ± 0·11 in group 2 and by 0·36 mmol/l ± 0·60 in group 3. In phase i HDLc decreased by 0·13 mmol/l ± 0·03 in group 1, by 0·31 mmol/l ± 0·03 in group 2 and by 0·18 mmol/l ± 0·04 in group 3, and in phase ii by 0·21 mmol/l ± 0·04 in group 1, by 0·29 mmol/l ± 0·03 in group 2, and by 0·26 mmol/l ± 0·04 in group 3. Comparisons between groups in each phase shows a relative decrease in C concentration attributable to the low-fat diet ($P < 0{\cdot}001$), but reducing dietary fat intake does not ameliorate the increases in T or decreases in HDLc due to isotretinoin.

A prospective study of the effect of isotretinoin on the sustainable rate of sebum secretion and the follicular reservoir in acne patients. B.R.HUGHES, J.GRAY AND W.J.CUNLIFFE, *Department of Dermatology, The General Infirmary, Leeds, U.K.*

The mode of action of isotretinoin in producing long-term remission in acne is poorly understood, but is thought to relate to the level of sebum secretion. Sequential measurements of sebum excretion over a 24-h period suggests that a reservoir of sebum exists in the follicular canals (Downing DT *et al. J Invest Dermatol* 1982; **79:** 226–8). This measurement, the follicular reservoir (FR), may be an independent variable to the sustainable rate of sebum secretion (SSER).

Using the method of Downing *et al.*, sebum was absorbed into bentonite clay over a 24-h period, extracted using ether and the weight of sebum calculated gravimetrically. Both the FR and SSER were then calculated from the data.

Twenty-two patients (mean age 19) were entered into the study. All had measurements performed prior to treatment with isotretinoin 1 mg/kg per day for 4 months. The patients were randomly divided and measurements performed on 10 patients immediately following completion of treatment and on the 12 remaining patients 1 year after treatment.

The mean SSER pretreatment was 0·73 $\mu g\ cm^{-2}\ min^{-1}$ and the FR 376 $\mu g\ cm^{-2}$. Immediately following treatment, the SSER was suppressed by 86% and the FR by 81%. However, 1 year following completion of treatment the SSER remained reduced at 43% of the original value and the FR suppressed by 64%.

These data suggest that the mechanism of action of isotretinoin in terms of long-term remission relates in part to the sebum excretion, but the greater suppression of the follicular reservoir could affect comedogenic factors in sebum.

Nail growth in disorders of keratinization (DOK) treated with acitretin. C.Reid, M.F.Corbett, W.A.D.Griffiths, *St John's Hospital for Diseases of the Skin, London, U.K.*

Finger-nails of psoriatic subjects grow faster than normal nails; the growth rate is further increased by the retinoid etretinate (Galosi A *et al.*, *Arch Dermatol Res* 1985; **277:** 138–40). The daily growth of normal finger-nails has been shown to be between 100 and 120 μm.

Finger-nail growth was studied in 15 patients with disorders of keratinization receiving acitretin. The group included five patients with keratoderma, five with pityriasis rubra pilaris, four with Darier's disease, and one with erythrokeratoderma progressiva. All patients received acitretin, 10–50 mg daily. The nails of both middle fingers and thumbs were measured by the method of Dawber (Dawber R, *Br J Dermatol* 1970; **82:** 454–7).

The daily rate of nail growth was calculated before treatment and after 0, 2, 6 and 12 weeks of treatment with acitretin. The mean pretreatment daily nail growth was 130·7 μm (95% confidence interval (CI) 99·2–162·2). Mean growth rates were increased at each time interval. The increase in growth rate was greatest during the first 2 weeks of treatment at 182·1 (CI 158·9–205·4). It remained elevated between weeks 2 and 6 at 161·2 (CI 130·7–191·7) and between weeks 6 and 12 at 140·0 (CI 111·2–168·7).

We have shown that: (i) There is an increased nail-growth rate in the four disorders of keratinization studied, and (b) Acitretin further enhances nail-growth rate and this effect is maximal during the first 2 weeks of treatment, returning towards the untreated value with further time on therapy. Whereas retinoids normalize the enhanced epidermopoiesis found in psoriasis, the nail apparatus responds paradoxically by more rapid growth (Galosi A. op. cit.). We have demonstrated that dissociation of target organ response to acitretin is seen in DOK.

The effects of β-adrenergic agonists and several peptidergic neurotransmitters on cAMP generation in cultured skin fibroblasts from psoriatic patients. D.J.Eedy,*† J.P.Canavan,‡ C.Shaw† and E.R.Trimble‡§, **Departments of Dermatology and §Clinical Biochemistry, Royal Victoria Hospital, Belfast, and †Departments of Medicine and ‡Chemical Pathology, The Queen's University of Belfast*

Vasoactive intestinal peptide (VIP), peptide histidine methionine (PHM), substance P and substance K have been identified in nerves in human skin but their action on skin fibroblasts is largely unknown. The aim of this study was to compare the effects of β-adrenergic agonists and putative peptidergic neurotransmitters on cAMP formation in skin fibroblasts from psoriatic and non-psoriatic individuals.

Fibroblast lines were established by the primary explant method from five control individuals and from lesional psoriatic skin from five patients. Fibroblasts, grown to confluency in Petri dishes, were incubated in the presence of isoproterenol (ISO), VIP, PHM, substance P and substance K. Basal cAMP levels were significantly lower in psoriatic than in control fibroblasts (4·5 ± 0·2 vs. 7·1 ± 0·6 pmol/mg protein respectively; $P < 0{\cdot}001$). Stimulation of cAMP generation by 10^{-8}–10^{-6} M ISO, in the absence or presence of the phosphodiesterase inhibitor 3-iso-butyl-1-methylxanthine (IBMX) was significantly less in psoriatic than in normal fibroblasts (10^{-6} M ISO 41·0 ± 5·7 vs. 199·5 ± 37·7 pmol/mg protein for psoriatic vs. controls respectively). Incubation with 10^{-6} M VIP, PHM, substance P and substance K, either in the presence or absence of IBMX failed to significantly increase cAMP levels in control or psoriatic fibroblasts.

These results indicate that cAMP generation is significantly reduced in psoriatic fibroblasts in response to β-adrenergic stimulation and that this effect is not due to enhanced phosphodiesterase activity. Under the conditions of this study the peptidergic neurotransmitters VIP, PHM, substance P and substance K had no effect on cAMP generation in skin fibroblasts.

Alterations in the microvasculature in the uninvolved skin of patients with psoriasis. M.ABDULLAH, S.BARTON AND R.MARKS, *Department of Medicine (Dermatology), University of Wales College of Medicine, Cardiff, U.K.*

Although it has often been stated that the apparently uninvolved skin of patients with psoriasis is abnormal and possesses the fundamental psoriatic fault, the evidence in favour of this concept is slim when critically evaluated. The present study has specifically investigated the dermal microvasculature component of the uninvolved skin of 20 patients (11 men, nine women, age-range 29–76 years) with plaque-type psoriasis of less than 20% body involvement untreated in the previous 4 weeks. Normal control subjects (five men, five women, age range 27–67 years) were also studied.

Biopsies were taken from sites 300 mm from the edge of lesions (also biopsied) in the patients and from comparable body sites in the controls. Sixteen 'step' sections (PAS stained) from each block were examined, and 70 fields examined according to a pre-determined systematic sampling procedure. A 100-point lattice in the eyepiece graticule was used to estimate the ratio of vascular to dermal elements in each field, using standard stereological procedures. The ratio of vascular endothelium to dermal tissue in psoriatic lesions was nearly twice that of controls (34·8 ± 10·1 × 10^{-3} compared to 8·9 ± 5·3 × 10^{-3}), and the vascular lumen three times greater (9·5 ± 2·4 × 10^{-3} compared to 3·07 ± 1·06 × 10^{-3}). These statistically significant differences were matched by significant differences between uninvolved and control samples for luminal volume only (4·93 ± 1·64 × 10^{-3} compared to 3·07 ± 1·16 × 10^{-3}). Some changes in endothelial and luminal volume may be explained by an increased number of vessels per unit volume (2·8 ± 1·0 involved, 2·2 ± 0·7 uninvolved, 1·7 ± 0·3 controls). Resting blood supply to the skin in the uninvolved sites determined by laser Doppler velocimetry is not significantly different compared to controls and does not match the structural alterations described.

These findings point to the endothelium as possessing a potentially important underlying fault in psoriasis.

Platinum complexes as potential anti-psoriasis agents. J.FEATHERSTONE, P.J.DYKES AND R.MARKS, *Department of Medicine (Dermatology), University of Wales College of Medicine, Heath Park, Cardiff, U.K.*

The platinum complex cisplatin (CP) has a proven record in the treatment of certain types of cancer. However, its use is limited by undesirable side-effects. Recently other platinum complexes such as JM8 and JM9, with fewer side-effects, have become available. Their main mechanism of action appears to be chelation with DNA with subsequent inhibition of synthesis. As such, these derivatives may be useful topically in the treatment of hyperproliferative skin conditions such as psoriasis. In this study the effects of platinum complexes on epidermal cells *in vitro* and *in vivo* have been examined.

When cultured human epidermal cells were exposed for 24 h to CP, JM8 and JM9 toxic effects were noted at 4 μM (CP), 10 μM (JM8) and 15 μM (JM9). Similar effects were noted when the experiments were repeated using cultured human fibroblasts. Tritiated thymidine labelling of epidermal cultures showed inhibition of DNA synthesis in CP treated cultures but inconsistent results for JM8 and JM9. Ultrastructurally all three compounds showed disruption of mitochondria as the predominant feature. Using tape-stripped hairless mouse skin an anti-proliferative effect of JM8 has been demonstrated (see Table 1).

These results show that platinum complexes are cytostatic for epidermal cells *in vivo* and *in vitro* and are potential candidates for the treatment of psoriasis.

TABLE 1. ($n = 10$ for all groups)

	Experiment (1)		Experiment (2)	
Treatments	Vehicle	1% JM8 aqu.	Vehicle	5% JM8 aqu.
Mitotic index	3·13 ± 2·12	0·82 ± 1·00	6·03 ± 3·22	0·00
Labelling index	9·92 ± 6·13	6·69 ± 2·51	14·9 ± 8·8	4·50 ± 3·78

Human monoclonal antibodies derived from virally transformed lymphocytes of a patient with bullous pemphigoid recognize epitopes associated with hemidesmosomes. H.SHIMIZU, T.HASHIMOTO,* T.NISHIKAWA* AND R.A.J.EADY, *Department of Cell Pathology, Institute of Dermatology, St Thomas's Hospital, London, U.K. and * Department of Dermatology, Keio University School of Medicine, Tokyo, Japan*

We and others have previously shown that autoantibodies (Ab) from patients with bullous pemphigoid (BP) bind to epitopes associated with hemidesmosomes (HD) in normal human skin (NHS), mainly at intracellular sites but also, to a lesser degree, outside the cells. Recently, we established two different human monoclonal antibodies (MAb) against the basement membrane zone (BMZ), designated 5E and 10D, derived from virally transformed lymphocytes from the peripheral blood of a BP patient (Hashimoto T *et al., J Invest Dermatol* 1988; **90:** 567). Immunoblotting showed that 5E but not 10D reacts with a 230 kDa protein from extracts of NHS. In the present study we set out to determine the ultrastructural binding sites of these novel MAbs.

By immunofluorescence both 5E and 10D were found to bind exclusively to the epidermal side of 1M NaCl-separated NHS. Pre-embedding immunoelectron microscopy (IEM) with immunoperoxidase was performed as previously described (Shimizu H *et al., Br J Dermatol* 1988; **119:** 717) using NHS as a substrate. 5E and 10D as well as BP-Ab were immunolocalized as discontinuous focal deposits along the BMZ of basal cells. For more precise immuno-localization, we used a post-embedding low temperature IEM technique. NHS was cryofixed in liquid propane at − 190°C, then cryosubstituted in 100% methanol at −80°C. The specimens were embedded in Lowicryl K11M at −60°C. Ultra-thin sections were incubated with 5E, 10D, BP-Ab or normal human serum, then anti-human IgG rabbit serum, followed by 5 nm gold conjugated Ab. With both 5E and 10D, as well as BP-Ab there was labelling mainly over the attachment plaques of HD and associated tonofilaments. Normal human serum used as a control showed no specific binding. These data indicate that both novel MAbs recognize epitopes associated with HD, in a similar distribution to the antigen labelled by BP-Ab, and should serve as valuable probes for further studies on BP antigen as well as HD.

A quantitative study of response to contact allergens alone and in combination. JANET MCLELLAND AND S.SHUSTER, *Dermatology Department, University of Newcastle upon Tyne, U.K.*

Multiple allergies are common and since the response to single allergens is log-dose related, we predicted that existing methods of patch testing would be inadequate to detect sensitivity to multiple allergens.

In 14 patients with positive patch tests to at least two unrelated antigens several dilutions in 20 μl were pipetted onto a Al-test patch both alone and with a second antigen then applied to the arm for 48 h. The 72-h responses were graded clinically and measured as change in skin thickness using Harpenden calipers. To confirm that the findings were applicable to standard methods of patch testing the allergens were diluted in paraffin in four patients.

The response was related to the log-dose of the allergen and skin thickness corresponded well with clinical grading. In six patients sub-threshold dilutions of both allergens were first established and when both allergens were combined on one patch measurable and clinical responses were produced. Using doses on the linear part of the response curve the response to the mixture was the sum of the responses to the individual components and at the plateau region it was less than the sum, as predicted by simple summation. Similar results were obtained with the allergens in paraffin. Six normal subjects produced no response to the allergens alone or in combination, excluding an irritant effect.

We have shown that the response to common antigens is log-dose related and that the response to multiple allergens is additive. Therefore contact dermatitis will occur by summation of responses to mixtures of allergens even when conventional patch tests are negative.

Cyclosporin A impairs the expression of established contact hypersensitivity. E.M.HIGGINS, J.MCLELLAND AND P.S.FRIEDMANN, *Dermatology Department, Royal Victoria Infirmary, Newcastle upon Tyne, U.K.*

Cyclosporin A (CsA) is known to be a potent blocker of the induction (afferent limb) of new immune responses. However, it is not yet clear whether it blocks the expression (efferent limb) of established immune responses. We have examined the effects of CsA on both the experimental elicitation responses and the clinical manifestations of allergic contact hypersensitivity. Five patients (three male, two female) with contact dermatitis due to allergic hypersensitivity to at least one antigen in the European standard patch test battery (ESB) were studied. Each subject was challenged with a series of dilutions (1/5–1/360) of the appropriate antigen (as used in the ESB) before and during therapy with CsA (5 mg/kg per day). Challenges were applied on standard patches (Finn chamber) to volar forearm skin for 48 h. Responses (increase in skinfold thickness) were measured at 72 h with Harpenden calipers. Responses to challenge were greatly inhibited by CsA in all five patients, with a shift of the dose–response curve to the right. Thus, the mean response to the 1/5 dilution fell from 1·7 mm (SE 0·5) to 0·4 mm (SE 0·14) ($P < 0{\cdot}05$, paired *t*-test). In addition, the median threshold dilution at which a positive response was elicited rose from 1/160 to 1/20. The changes in response to antigen challenge were matched in all five patients by a virtually complete resolution of the chronic allergic contact eczema. In conclusion, the efferent limb of established allergic responses is clearly inhibitable by CsA in moderate therapeutic dosage.

Accessory function of keratinocytes. A.C.CHU AND J.MORRIS, *Unit of Dermatology, Hammersmith Hospital, London, U.K.*

A SV40 transformed keratinocyte cell line, SVK14, was used to examine the possible accessory cell function of keratinocytes.

Using radio-iodinated recombinant hepatitis B surface antigen, SVK14 cells were shown to take up the antigen and release trichloracic acid soluble (low molecular weight) protein after incubation in a time-related fashion. Polyacrylamide gel electrophoresis and autoradiography of SVK14 cells after periods of incubation with the antigen showed loss of high molecular weight bands and the appearance of low molecular weight bands.

When SVK14 cells were stimulated with γ interferon, HLA-DR and ICAM1 were expressed by the cells in a dose dependent fashion. DR^+ SVK14 cells failed to stimulate alloreactive or antigen specific T-cell clones, but DR^+ and DR^- SVK14 cells were able to stimulate allogeneic T cells, which was not due to cytokine release by the cells. This suggests that SVK14 cells are able to stimulate an allogeneic response via Class I antigen.

When DR^+ SVK14 cells were used to stimulate alloreactive T-cell clones, they induced long-term non-reactivity in these clones, which could not be further stimulated by conventional accessory cells.

The results of these studies indicate: (i) keratinocytes can process antigen. (ii) Keratinocytes can stimulate an allogeneic response via Class I antigen. (iii) DR^+ keratinocytes can not stimulate an antigen specific or alloreaction in T-cell clones. (iv) Keratinocytes may be able to induce tolerance in T cells.

Quantitative evaluation of melanoma cell invasion in three-dimensional confrontation cultures *in vitro* using automated image analysis.

J. SMOLLE, C. HELIGE,* H. KERL, H. TRITTHART,* AND H. KRESBACH, *Department of Dermatology and *Department of Medical Physics, University of Graz, Austria*

Tumour-stroma confrontation systems provide models for tumour-cell invasion *in vitro*. In contrast to proliferation and cytotoxicity, however, quantitative assessment of tumour invasion has largely been unsuccessful up to now. In this study, we present an image analysis program, which yields a reliable quantitative description of melanoma cell invasion in an *in vitro* model.

K 1735 M2 and K 1735 CL16 melanoma cell variants were cultured as multicellular spheroids and confronted with embryonic chick heart fragments for 2–196 h. At different time intervals, cryostat sections were stained with an antiserum directed to embryonic chick heart. Image analysis was based on automatic grey level processing and binary image operations using a computerized image analysis system (Vidas; Zeiss, F.R.G.) producing a total of 39 morphometric and densitometric parameters. Ten index slides were used as a test set.

On Spearman's rank correlation test, 25 out of 39 parameters correlated with the reference value of invasion ($P = < 0.05$), which was derived from the semi-quantitative evaluation of five independent observers. Two parameters combining the stroma outline and the total amount of stroma tissue completely reproduced the judgement of the morphologists in the test set ($P = < 0.0001$).

Our study shows, that objective definition and measurement of melanoma cell invasion *in vitro* is possible. The quantitative descriptors provide a useful tool for investigating biological and pharmacological modifications of the particular confrontation model.

Evidence of increased receptor 'cross talk' in B16 melanoma cells of high metastatic potential.

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A positive association between metastatic potential and cyclic AMP responsiveness to agonist stimulation has been noted for B16 melanoma cells (Sheppard *et al.*, *Int J Cancer* 1986; **317**: 713–22). Our aim was to investigate how the cyclic AMP system of these cells differed and how this cyclic AMP signal related to melanogenesis.

F10C1 cells produced the greatest number of lung foci 18 days post-i.v. injection in C57BL mice and had the greatest cyclic AMP response to agonist stimulation (137 fold over basal). However, these cells had the least tyrosinase activity and melanin production *in vitro*. Conversely, cells producing the least lung foci (F1C29) had the least cyclic AMP response (14 fold over basal) but the greatest tyrosinase activity and melanin production *in vitro*.

Adenylate cyclase activities were similar in broken cell preparations of F10C1 and F1C29 cells irrespective of the agonist used, as was the distribution of cyclic AMP between the intracellular and extracellular compartments. In intact cells however, F10C1 cells had high cyclic AMP accumulation which was unaffected by treatment of cells with the protein kinase C activator PMA (from 1 to 1000 nM for 30 min) but the protein kinase C antagonist H7 decreased cyclic AMP accumulation. Conversely, F1C29 cells responded to PMA (100 nM) with increased cyclic AMP accumulation to agonist stimulation (153, 145, 127% of control in three consecutive experiments). H7 was without effect on these cells but inhibited the response to PMA.

We conclude highly metastatic B16 cells may produce more agonist-stimulated cyclic AMP due to a positive endogenous stimulatory effect of protein kinase C on the adenylate cyclase enzyme. This same effect can be duplicated pharmacologically in B16 cells of low metastatic potential but does not occur spontaneously. Further, the high cyclic AMP generated does not appear to be coupled to melanogenesis for highly metastatic B16 cells.

Evidence for a calcium/calmodulin inhibitory control of melanogenesis *in vitro*. J.BUFFEY, S.S.BLEEHEN* AND S.MACNEIL, *Department of Medicine, Northern General Hospital and *Department of Dermatology, University of Sheffield, Royal Hallamshire Hospital, Sheffield, U.K.*

Recent work from this laboratory has shown that B16 melanoma cell lines, which vary in their ability to pigment to MSH, all show significant cAMP accumulation in response to MSH *in vitro* and produce equally well pigmented tumours *in vivo*. A stimulatory effect of calmodulin antagonists (Hill SE *et al.*, *Pigment Cell Research* (in press)) and an inhibitory effect of calcium ionophore on melanoma cells have been found (Fuller, *Pigment Cell Research* 1987; **1:** 176–80) suggesting that calcium may play an inhibitory role in the control of melanogenesis.

Our aim in this study was to further investigate a possible role for Ca in the control of melanogenesis. We used drugs reported to affect the intracellular Ca signalling system; the Ca ionophore A23187 to increase intracellular Ca; TMB8 [8-(*N*,*N*-diethylamino) octyl 3,4,5-trimethoxy-benzoate hydrochloride] to reduce intracellular free Ca and 5 iodo C_8 (*N*-(6-aminooctyl)-5 iodo-1-naphthalene-sulphonamide) to inhibit calmodulin activity (see Hill SE *et al.*). B16 F1 cells seeded at 18×10^3 cm^2 showed significant inhibition of melanogenesis ($7·4 \pm 1·6$ for control to $3·6 \pm 0·6$ pg melanin/cell, $n = 5$ experiments, $P < 0·05$) when cultured with 2 μM A23187 for 2 days. Five iodo C_8 (25 μM) significantly increased melanin from $6·4 \pm 1·3$ to $9·6 \pm 1·8$ pg melanin/cell ($n = 4$ experiments, $P < 0·05$) over this period as did TMB8 (10 μM, from $4·6 \pm 1·6$ to $9·8 \pm 1·8$ pg melanin/cell, although the latter did not achieve statistical significance, $n = 5$ experiments). In comparison, the maximum melanogenesis achieved with MSH (10^{-6} M) in the same experiments was from $7·34 \pm 1·6$ to $14·3 \pm 2·5$ pg melanin/cell, $n = 5$ experiments.

These data are consistent with a calcium/calmodulin inhibitory control of melanogenesis functioning in these cells at high density. Also the melanogenic response to these agents was dependent on cell density, suggesting that such calcium-inhibitory control of melanogenesis varies with cell density.

α-MSH causes a rise in cyclic AMP in human melanocytes with no effect on basal or ultraviolet-induced melanogenesis. P.S.FRIEDMANN, F.WREN, J.BUFFEY* AND S.MACNEIL*, *Dermatology Department, Royal Victoria Infirmary, Newcastle upon Tyne, and *Department of Medicine, Northern General Hospital, Sheffield*

α-MSH is known to stimulate melanogenesis in murine melanocytes and melanoma cells via a rise in cyclic AMP (cAMP). There is controversy regarding the role of α-MSH in human melanocyte responses so we have examined its effects on levels of cAMP, melanin content and response to ultraviolet radiation (UVR) in cultured human melanocytes (HuMC). Foreskin HuMC were cultured by the method of Gilchrest (independent of TPA) (*J Invest Dermatol* 1984; **83:** 370–6). Following addition of α-MSH (10^{-6} M) there was a rise in cAMP levels maximal between 5 and 15 min: control $3·6 \pm 0·7$ pM/10^5 cells, with α-MSH $9·4 + 3·2$ pM/10^5 cells. After 7 days culture in the presence of α-MSH (10^{-8}–10^{-6} M) melanin content increased only 35%. By contrast, Forskolin (10^{-5} M) induced a 9·5-fold rise in cAMP after 5 min and a 10·9-fold rise in melanin content after 7 days. When HuMC were irradiated daily for 6 days with UVR (Helarium fluorescent lamps emitting 20% UVB, 80% UVA) melanin content rose 2·7-fold (SE 0·3). This was unchanged or slightly reduced in the presence of α-MSH (10^{-8}–10^{-6} M). Parallel observations on Cloudman S91 melanoma cells showed α-MSH caused only an 80% increase in melanin content after 4 days. The rise in melanin content induced by 3 daily UV-irradiations (2·4 fold, SE 0·5) was unchanged by α-MSH (10^{-8}–10^{-6} M). In conclusion, although α-MSH induces a small rise in cAMP in HuMC this does not result in melanogenesis. Furthermore, the response to UVR is not affected by α-MSH in either HuMC or S91 cells.

Isolated human hair follicle dermal papillae induce hair growth in athymic mice. K.A.HORNE, J.C.FORRESTER* AND C.A.B.JAHODA, *Department of Biological Sciences, University of Dundee and *Department of Surgery, Ninewells Hospital and Medical School, Dundee, U.K.*

The induction properties of the rat vibrissa follicle dermal papilla have been well documented but, up to now, the same capacity for stimulating hair growth has not been demonstrated experimentally for the human follicle dermal papilla. We have grafted human papillae into athymic mice to determine whether they have the ability to induce fibre production from inactivated follicles.

Dermal papillae were dissected from human hair follicles in minimum essential medium, and implanted into the epidermal cavities of athymic mouse-whisker follicles which had been isolated, and prepared by amputation of their lower halves, and removal of their fibres. These composite grafts were then introduced into granulation tissue pockets, formed by subcutaneous implantation of glass discs in the backs of athymic mice, two weeks previously.

Macroscopic observation revealed that four out of 20 operational follicles had resumed hair growth when recovered up to 6 weeks post-operatively. Histology showed that at their bases, dermal papillae had induced the formation of epidermal matrices which were actively forming fibres (in one case in duplicate). Importantly, in many other follicles, papillae which had not remained in optimal position to interact with epidermis were still visible. In these cases failure to induce hair growth could therefore reasonably be attributed to technical complexities.

Eleven control grafts of upper follicles without dermal papillae did not produce hair, and showed no histological signs of renewed bulbar organization. This demonstration that the adult human dermal papilla can induce hair growth provides much needed biological justification for the recent upsurge of work on cultured human dermal papilla cells. It also suggests the recognition of common messages by tissues (a) from recognizably distinct hair types and (b) from different species, as mouse vibrissa follicle epidermis has responded to human body follicle dermis. This result also helps vindicate our combined use of animal models and culture systems for hair growth studies.

Co-culture of rat dermal papilla and epidermal cells—absence of proliferative stimulation.

S.M.Heron and C.A.B.Jahoda, *Department of Biological Sciences, University of Dundee, Dundee, U.K.*

Previous studies have described growth stimulation of rat skin basal epidermal cells cultured directly on top of living or mitomycin-treated rat vibrissa dermal papilla cells. This work was aimed at determining whether similar enhancement of proliferation could be achieved by growing epidermal cells on membrane filters raised above living dermal cells.

Papilla cells or fibroblasts from adjoining mystacial pad skin at passage 1–3 were plated at a density of 7×10^4 cells in 35-mm culture dishes (Falcon). Basal epidermal cells isolated from 1-day-old rats were added after 24 h (growth phase effect) or 7–14 days later after dermal cells had become confluent (stationary effect). They were seeded into duplicate, membrane supporting chambers (Millicell HA, Millipore) (5×10^4 cells/chamber) above the established papilla or fibroblast cells, or over plastic substrate as a control. The co-cultures were grown for 7 days in minimal essential medium (Eagle) with 20% fetal calf serum (FCS), then pulsed for 4 h with [^{3}H]-thymidine (2 μCi/ml) for scintillation counting.

Labelling of the whole co-culture system permits two-way effects to be studied. Replicate ($n = 8$) experiments have shown that epidermal cells cultured over growth or stationary phase papilla cells display no increase in thymidine incorporation compared to those above plastic. Epidermal cells over growth phase fibroblasts incorporate twice as much label as controls above plastic. Epidermal cells over stationary phase fibroblasts show less increase in label uptake. In the reverse direction no significant effects of epidermal cells on papilla or fibroblast cell labelling were detected. Counts were at equivalent levels to control cells grown without epidermal influence.

Dermal papilla cells do not appear to retain growth promoting effects on basal epidermal cells at a distance—there is no obvious medium conditioning. The low levels of epidermal stimulation produced by dividing fibroblasts might be an epidermal response to fibroblast autocrine factors in the medium.

Hair follicle destruction and regeneration in guinea-pig skin after cutaneous freeze injury.

S.M.Burge and R.P.R.Dawber, *Department of Dermatology, Slade Hospital, Oxford, U.K.*

The loss of hair following cryosurgery for benign and premalignant skin lesions using single-freeze schedules is frequent but unpredictable; with more aggressive malignancy schedules hair loss is the rule. The former often occurs without clinical scarring, suggesting a different mechanism than the latter, which appears to relate to overall pathological scarring. Since cryosurgery is an increasingly important mode of treatment it is important to understand the mechanisms of freeze injury on normal as well as pathological skin.

We have therefore carried out histological studies on the hair follicles of the skin of nine guinea-pigs after cryosurgery injury using the standard liquid nitrogen spray techniques and times used in clinical practice. For standardization, the cone spray method was employed, liquid nitrogen being sprayed into the neoprene cone applied to the skin; after ice formation, spraying was continued for 10, 25, and 30 s at different flank sites. Biopsies were taken at 2 days, 1 week, 2 weeks, 1 month and 6 months after the freeze. Specimens were stained with routine haematoxylin and eosin or Verhoeff's elastic van Gieson.

Within the period of the observations, all areas showed alopecia. The 10-s freeze specimens showed very little follicular inflammatory change with rapid epidermal healing but follicular and sebaceous gland loss. Follicles were either shed at the surface or destroyed *in situ*—suggesting some form of autolytic process in view of the absence of surrounding dermal tissue reactivity and the preservation of the follicular connective tissue sheath. No perifollicular granulomatous inflammation was seen. The 25- and 30-s freeze specimens all showed considerable dermal damage, the follicles being shed as part of the eschar formed from the necrotic tissue; after healing no specimens showed evidence of residual follicles. In none of the treatment schedules was dermal-papilla preservation or regeneration noted, this presumably being the major factor in the failure of hair to regrow.

Inhaled corticosteroids and skin thinning. D.SHUTTLEWORTH, C.EDWARDS, S.CAPEWELL,* S.REYNOLDS* AND A.Y.FINLAY, *Department of Medicine (Dermatology), University of Wales College of Medicine Cardiff, and *Department of Thoracic Medicine, Llandough Hospital, Cardiff.*

Although complications can arise following treatment with high-dose inhaled corticosteroids, (ICS), there have been no studies which have attempted to quantify the cutaneous effects of this therapy. The aim of the study was to compare skin thickness in: (i) patients using high-dose ICS therapy; (ii) normal controls; (iii) patients taking systemic prednisolone for pulmonary disease; (iv) patients treated with low-dose inhaled ICS.

Skin thickness was measured at three sites using a pulsed A-scan ultrasound device as follows: (i) dorsum of right hand; (ii) upper inner right arm, and (iii) upper outer quadrant of left buttock.

Solar elastosis was assessed on the face and hands using a 10 cm visual analogue scale and patients were assessed as having 'normal' or 'high' sun-exposure on the basis of previous history. A history of skin fragility and spontaneous bruising was sought. Patients were examined for the presence of striae and purpuric/ecchymotic lesions. A total of 68 patients have been studied as follows: group a, $n = 21$; group b, $n = 17$, group c, $n = 14$; group d, $n = 16$.

Skin thickness was significantly reduced for all sites measured in patients taking oral steroids (group c) compared with normals (group b) ($P < 0{\cdot}05$). Similarly, skin thickness was also significantly reduced for all sites measured in the group of patients using high dose ICS therapy (group a) compared with normals (group b) ($P < 0{\cdot}05$, Mann–Whitney *U*-test). There was no difference between skin thickness in patients using low-dose ICS therapy (group d) when compared with normals (group b). The four groups were well matched for age, sex, previous sun-exposure (with the exception of patients in group c, who had lower than average exposure) and the degree of solar elastosis. Skin fragility was more frequently reported in groups a and c, ($P < 0{\cdot}001$, χ^2 test) compared with controls. Purpuric lesions were also more commonly found in these groups when compared with controls, ($P < 0{\cdot}02$ and $P < 0{\cdot}001$). Patients in group d did not differ significantly from normal controls. No difference was found in the frequency of striae. This study has demonstrated that high dose ICS therapy is associated with significant objective cutaneous thinning and clinical fragility.

Mediators of Ro/SSA antigen expression on cultured epidermal keratinocytes. S.K.JONES, *Department of Dermatology, Bristol Royal Infirmary, Bristol, U.K.*

Anti-nuclear antibodies are useful for identifying connective tissue diseases and subsets within these diseases. Evidence that they may also be pathogenic comes from the neonatal lupus syndrome, where the presence of Ro/SSA (Ro) antibodies relates to clinical disease, and from the finding that the Ro antigen is found on keratinocyte cell surfaces after ultraviolet (UV) exposure where it can bind circulating antibody. This study investigates which wavelengths of light (UVA or UVB) are responsible for surface expression, and whether other stimuli induce or modify it.

Human keratinocyte cultures were grown in MCDB 153 on Lab-Tek slides. Test substances included γIF (10 100 and 500 U/ml), cholera toxin (2·5 nM), phorbol-12-myristate-13-acetate (PMA) (1 and 10 ng/ml), hydrogen peroxide (10^7 and 10^8 M), leukotriene B_4 (10^{-8}, $^{-9}$, $^{-10}$ M), dihydrotestosterone (1, 10 and 50 ng/l), progesterone (10^{-7}, $^{-8}$, $^{-10}$ M) and 17β oestradiol (10^{-6}, $^{-7}$, $^{-8}$ M). After incubation in the above, some slides were also exposed to UVA or UVB. Cells were then assessed for viability and surface Ro expression, the latter by the binding of human Ro antibodies detected by immunofluorescence. Each experiment was carried out in triplicate.

Cell surface expression was induced by UVB but not by UVA, this being increased from 10 to 20% by prior incubation with 17β oestradiol. Seventeen β oestradiol did not produce expression on its own. These findings would support the role of UVB in the induction of cutaneous lupus lesions and may help to explain the increased incidence of lupus in female patients.

The induction of heat-shock proteins in human skin cells. M.J.EDWARDS, M.R.O'DONOVAN,* P.J.DYKES, V.MERRETT, H.E.MORGAN* AND R.MARKS, *Department of Medicine (Dermatology), University of Wales College of Medicine, Cardiff U.K. and *Boots Pharmaceutical Research Department, Nottingham, U.K.*

All organisms so far examined contain heat-shock proteins (HSP) which are synthesized in response to heat and other stress-inducing environmental agents. The cellular functions of the heat-shock proteins have not been fully defined. However, they are thought to enable cells to survive toxic changes in their environment (Lindquist S, *Ann Rev Biochem* 1986; **55:** 1151–91). Most of the 70 kDa stress-induced gene family are expressed constitutively in cultured human cells. However, HSP72 (72 kDa heat-shock protein) is induced by physiological change in the cells' environment and may be used as a marker to toxicity.

We have investigated the heat-induced synthesis of HSP72 through the cell cycle of cultured human skin fibroblasts and keratinocytes. Following exposure to heat (43°C for 90 min) newly synthesized HSP72 was detected by SDS–PAGE and immunofluorescence within 2 h of exposure in the cytoplasm of fibroblasts and keratinocytes. The newly synthesized HSP72 then migrated into the nucleus and localized in the nucleolus, where it remained for a further 4–6 h. Our results are consistent with those reported in 1984 (Welch WJ, Feramisco JR. *J Biol Chem* 1984; **259:** 4501–13), using cultured mammalian cells.

We have also measured the synthesis of HSP72 in cultured human keratinocytes and fibroblasts following exposure to heavy metals, genotoxic chemicals, and detergents such as sodium lauryl sulphate. We are assessing the suitability of HSP72 induction and localization within the nucleolus as a marker for the detection of toxic xenobiotic agents.

Changes in intracellular free calcium of human keratinocytes during differentiation and stimulation with EGF. G.R.SHARPE, J.I.GILLESPIE* AND J.R.GREENWELL*, *Department of Dermatology, and *Department of Physiological Sciences, University of Newcastle upon Tyne, U.K.*

Intracellular free calcium ($[Ca^{2+}]_i$) is an important second messenger and regulates many cell processes. Microspectro-fluorimetric techniques now permit the measurement of $[Ca^{2+}]_i$ within living cells. Human keratinocytes were grown on glass coverslips in the serum-free medium MCDB153 with an extracellular calcium concentration ($[Ca^{2+}]_o$) of 70 μM. $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent dyes FURA-2 and INDO-1. Cells were loaded by incubation with the acetoxymethyl esters of the dye. Measurement of fluorescence emitted from single cells was made using a NIKON DIAPHOT inverted microscope with either a dual emission or dual excitation photon counting system (Newcastle Photometric Systems).

Cells grown in 70 μM $[Ca^{2+}]_o$ form a proliferating monolayer but on increasing the calcium to 1 mM there is progressive differentiation and stratification with a reduction in cell growth. $[Ca^{2+}]_i$ in low calcium culture was 100 ± 1·3 nM (mean ± SEM) and increasing $[Ca^{2+}]_o$ from 70 μM to 1 mM caused an early rise in $[Ca^{2+}]_i$ complete by 2 h (133 ± 11 nM). The increase in $[Ca^{2+}]_i$ preceded the morphological changes and growth inhibition (thymidine labelling studies) induced by increasing $[Ca^{2+}]_o$. Heterogeneity of $[Ca^{2+}]_i$ within cultures was demonstrated. A clear difference was seen between basal and layered differentiated cells. The $[Ca^{2+}]_i$ in spontaneously-differentiated cells of low Ca^{2+} cultures (372 ± 19 nM) was similar to that of Ca^{2+} induced differentiated cells. These observations are consistent with an increase in $[Ca^{2+}]_i$ mediating differentiation of human keratinocytes.

Cells grown in the absence of EGF for 8 days showed similar $[Ca^{2+}]_i$ to cells grown in complete medium (EGF 10 ng/ml). However on replacement of EGF there was a rapid and large rise in $[Ca^{2+}]_i$ within 1 min of the addition. The duration of the peak in $[Ca^{2+}]_i$ was less than 4 min with values returning to resting levels. The response to EGF is in contrast to the slow sustained rise seen with an increase in $[Ca^{2+}]_o$.

Rapid increases in keratinocyte intracellular free calcium-induced by 1α-25-dihydroxy vitamin D_3. B.BITTINER, S.S.BLEEHEN AND S.MACNEIL*, *Departments of Dermatology, Royal Hallamshire Hospital and *Medicine, Northern General Hospital, Sheffield University, Sheffield, U.K.*

1α-25-dihydroxyvitamin D_3 (DHCC) has been shown to be of benefit in psoriasis when administered orally or topically. Although DHCC has an anti-proliferative effect on human keratinocytes and promotes differentiation, its mechanism of action is unclear. Calcium and calmodulin play an important role in the proliferation and differentiation of keratinocytes (Al-Ani AM *et al.*, *Br J Dermatol* 1988; **119:** 295–306), and calmodulin is elevated in psoriatic keratinocytes. Further, recent work has shown that DHCC acutely increases intracellular calcium in B15 melanoma cells (Hill SE *et al.*, *Br J Dermatol* 1989; **120:** 21–30). There is no equivalent published data using DHCC in human keratinocytes.

We have grown primary cultures of keratinocytes from neonatal foreskins on collagen coated glass coverslips and measured the intracellular calcium using the fluorochrome FURA-2. DHCC increased calcium concentration in 18 out of 18 consecutive slides from five donors. Whereas the calcium ionophore ionomycin produced an immediate, sustained increase in calcium, a range of responses was seen to DHCC: in 10/18 there was a transient response to DHCC lasting for 6–60 s, which developed within a few minutes; in four there was a sustained increase in calcium; in the remaining four there was a sustained response with superimposed transient responses. Quantitation of the intracellular calcium was not feasible in all instances, but where possible yielded a resting value of 369 ± 50 nM ($n = 14$), which increased to 692 ± 276 nM ($n = 4$) with 10^{-10} M DHCC and 626 ± 276 nM ($n = 8$, $\bar{x} \pm$ SEM) with 10^{-8} M DHCC.

In summary, we have demonstrated that DHCC is capable of increasing free intracellular calcium in normal human keratinocytes. The significance of this relatively acute effect of DHCC on intracellular calcium on the proliferation and differentiation of normal and psoriatic keratinocytes requires further investigation.

Early induction of ornithine decarboxylase activity by hexadecane in neonatal pig skin is independent of protein kinase C activation. S.OWUSU, E.J.WOOD AND W.J.CUNLIFFE, *Department of Biochemistry, University of Leeds and Department of Dermatology, Leeds General Infirmary, Leeds, U.K.*

Topical application of hexadecane to rodent skin has been shown to induce hyperproliferation and hyperkeratosis similar in some respects to the hyperproliferation and hyperkeratinization observed in conditions such as psoriasis, pityriasis rubra pilaris, and Darier's disease. Polyamines are essential for cell proliferation and ornithine decarboxylase (ODC), the rate-limiting enzyme of polyamine biosynthesis, is rapidly induced by many external stimuli. This induction is one of the important biochemical changes occurring at an early stage when quiescent cells are transformed to the growing state. The application of hexadecane to neonatal pig skin resulted in rapid biphasic rise in the level of ODC activity, the early phase of activation being independent of protein synthesis. Hexadecane also markedly stimulated [^{3}H]-thymidine incorporation and this stimulation was almost totally abolished by the specific ODC inhibitor, α-difluoromethylornithine, indicating that ODC is involved in the hexadecane-induced hyperproliferation. However, the intracellular mechanisms leading to the stimulus-induced ODC activity are still not clear. The calcium/phospholipid-dependent protein kinase C (PK-C) associated with cell membranes appears to play a vital role in intracellular processes including tumour promotion. Pig epidermal PK-C is also known to be activated by hexadecane within 10 min of application. However, we found that the specific and potent PK-C inhibitors, staurosporine and H-7, failed to inhibit the early rise in hexadecane-induced ODC activity but caused a marked decrease in the second, protein synthesis-dependent phase. The result indicates that PKC may enhance the synthesis of ODC as previously reported but probably does not have any effect on the activation of the latent ODC.

Differential expression of the α and β retinoic acid receptors. J.L.REES AND C.P.F.REDFERN, *Dermatology Department and Medical Molecular Biology Group, University of Newcastle upon Tyne, U.K.*

Retinoids exert profound effects on epithelial cell development and differentiation both *in vivo* and *in vitro*. The cloning of cDNAs coding for two retinoic acid receptors (RAR-α and RAR-β) has recently been reported. The predicted primary structures of these RARs have considerable homology with each other and with the steroid/thyroid family of DNA binding proteins. We have previously shown (Rees JL, Redfern CPF, *Biochem J*, 1989; **259:** 917–19) that these receptors are widely distributed but have distinct distributions not specific to epithelial tissues. In the present study we show that RAR-α and RAR-β transcripts are expressed in rat and human skin, and in dermal fibroblasts and keratinocytes *in vitro*. RAR-α transcripts were expressed in all tissues but were more abundant in dermis and dermal fibroblasts than in epidermis or keratinocytes. RAR-β mRNA was expressed in skin but patterns of expression differed between rat and human samples. In human skin, dermal fibroblasts and keratinocytes, two RAR-β transcripts (ca. 3·3 and 3·4 kb) were expressed. Conversely in rat skin, dermal fibroblasts and keratinocytes only the smaller transcript was detectable and this was more abundant in cultured cells than in whole tissue. The relatively high level of expression of RAR-α and RAR-β in dermis and dermal fibroblasts suggests that the effects of retinoic acid on the dermis may be as important as those on the epidermis. Finally we have investigated the influence of changes in differentiation of cultured keratinocyte induced by calcium shifts on receptor expression.

Analysis of *ras* gene mutations in non-melanoma skin cancer by the polymerase chain reaction (PCR) and mutation-specific oligonucleotide probing. S.I.WHITE AND A.BALMAIN*, *Department of Dermatology, University of Glasgow, and *Beatson Institute for Cancer Research, Glasgow, U.K.*

Point mutations in all three *ras* genes, Harvey, Kirsten and 'N', have been identified in a variety of human tumours. Irrespective of the gene the mutation will occur at codon 12, 13 or 61. The PCR amplification of DNA and probing with mutation-specific oligonucleotides means that a large series of tumours can be efficiently screened for a point mutation.

DNA was extracted from 32 basal cell carcinomas (BCCs) and seven squamous cell carcinomas (SCCs). PCR amplification was performed for the regions of DNA around Harvey-*ras* codons 12, 13 and 61, and Kirsten-*ras* codons 12 and 61 using the heat stable Taq polymerase enzyme. The amplified DNA was probed with ^{32}P-ATP labelled mutation specific oligonucleotides for the respective *ras* gene codons.

Six of the BCCs and three of the SCCs had a mutation in codon 12 of the Harvey-*ras* gene. In each case the mutation was a G to T transversion at the second base (wild type GGC, mutation GTC). All the other mutations for the *ras* genes were negative. To investigate the possibility of a germ line mutation white blood cell DNA from 22 of these patients was similarly amplified and probed. No mutation was found in any of the WBC DNAs.

A G to T transversion is unlikely to be induced by ultraviolet light but is frequently induced by chemical carcinogens. This single point mutation would be compatible with non-melanoma skin cancer being induced by a chemical carcinogen and promoted by ultraviolet light.

Cloning, sequencing and chromosomal localization of human keratin 17. P.E.Bowden, N.C.Popescu, J.Doniger and J.A.DiPaolo, *Laboratory of Biology, NCI, NIH, Bethesda, MD, U.S.A.*

Keratins are epithelial-specific intermediate filament proteins. Although 19 human keratins (HK1–HK19) have been described, eight additional hair-specific keratins have recently been identified. Several members of this multi-gene family are major differentiation products of mammalian epidermis. Two of these, HK16 and HK17, are difficult to resolve by protein gel electrophoresis. Thus, while keratins of this size are expressed in hyperproliferative skin disorders, cultured keratinocytes, hair follicles, viral-transfected epithelial cells and tumour cells, the precise differential expression of HK16 and HK17 is still unclear. Keratin cDNA's and genes have unique properties so that the expression of HK16 and HK17 can be studied. To facilitate these studies, we have isolated several keratin clones and the present results describe the cloning of HK17.

A human epidermal cDNA library was screened with a mouse keratin 14 cDNA (Roop *et al. Cancer Res* 1988; **48:** 3245) and the partial human keratin 14 cDNA was isolated and sequenced. A cDNA library (HPV-transfected keratinocytes) and a human cosmid genomic library were screened with the HK14 cDNA. An almost full-length clone was obtained for HK17 (Bam HI 1400 bp fragment) and several clones containing type I keratin genes (including HK14, HK16 and HK17) were obtained. The HK17 cDNA was subcloned into pGEM 3 and sequenced by the oligo-primed double-stranded dideoxy method. The sequence obtained begins in helix 1A of the protein and extends through the stop codon and 3′ non-coding sequences to the poly-A tail. The sequence is similar to that of HK14 and HK16 in the conserved helical regions but as with other keratins, the C-terminal and 3′ non-coding sequences are unique. A specific probe was made to the unique 3′ end of the HK17 cDNA and subcloned. Northern blotting demonstrated that HK17 was prominent in HeLa cells (HK16 absent), cervical epithelium, cervical tumours and cultured human keratinocytes. Chromosomal *in-situ* hybridization with the HK17 specific probe showed that the HK17 gene was localized to chromosome 17 (17p12 and 17q12), regions that contain other type I keratin genes. Preliminary mapping of the cosmid clones also indicated that the genes for HK14, HK16 and HK17 are closely linked. Thus, we have cloned another human type I keratin (HK17), shown that the sequence is similar to other type I keratins and unique at the C-terminus (3′ end). Furthermore, the HK17 gene is localized to two separate regions of chromosome 17, one on the short arm and one on the long arm.

The relationship between chronological age and erythemal response to UVB irradiation. N.H.Cox, P.M.Farr and B.L.Diffey*, *Dermatology Department, University of Newcastle upon Tyne, and *Regional Medicine Physics Department, Dryburn Hospital, Durham, U.K.*

Irradiation with UVB has been reported to cause less erythema and smaller increases in levels of inflammatory mediators in the skin of elderly compared with young subjects (Gilchrest BA *et al.*, *J Invest Dermatol*, 1982; **79:** 11–15). However, the erythemal response to UVB irradiation has not previously been measured in different age-groups. We have constructed dose–response curves for UVB-induced erythema in 14 subjects aged over 60 years and 11 subjects aged 16–20 years.

Nine adjacent 1 cm sites on the mid-back were irradiated with a series of doses of UVB (maximum 56 mJ/cm^2) using a filtered mercury arc lamp. Erythemal responses were measured in triplicate before and 24 h after irradiation using a reflectance instrument; the minimal erythema dose (MED) was assessed visually. There was no difference between the MEDs in the older (geometric mean 11·9 mJ/cm^2, range 6·7–21·9) compared with the young age group (geometric mean 12·2 mJ/cm^2, range 7·6–21·9). The measured erythema at each site was compared with the pre-irradiation measurement to calculate the increase in erythema index (ΔE), which increased linearly with log UVB dose. Slopes of the dose-response curve were calculated for each patient using linear regression. There was no difference between the mean (± 1 SD) slope in the older group (0·322 ± 0·114) and the young age group (0·322 ± 0·053), although the range of values increased with age (older group 0·175–0·588, young group 0·240–0·410). A hypothetical 'threshold' dose can be calculated by extrapolation of the linear dose-response curve to $\Delta E = 0$. Threshold doses in the older group (geometric mean 6·9 mJ/cm^2, range 4·4–16·9) were not significantly different to the young group (geometric mean 8·3 mJ/cm^2, range 4·0–18·3).

We conclude that the 24 h erythemal response to UBV does not significantly alter with increased age.

Inhibition of photosensitivity in erythropoietic protoporphyria with terfenadine. P.M.Farr, B.L.Diffey* and J.N.S.Matthews†, *Departments of Dermatology and †Medical Statistics, University of Newcastle upon Tyne and *Regional Medical Physics Department, Dryburn Hospital, Durham, U.K.*

The acute symptoms of photosensitivity in erythropoietic protoporphyria (EPP) are similar to those of solar urticaria, a disorder which is mediated in part by histamine and responds to treatment with terfenadine. We have studied the effect of terfenadine on the cutaneous response to irradiation with blue light in patients with EPP, before and during conventional treatment with β-carotene.

A mercury arc lamp, filtered to isolate only blue light, was used to irradiate six 10-mm diameter sites on the back of each of seven male patients with EPP (age range 9–43 years) using doses ranging from 1·5 to 48 J/cm^2. The maximum diameter of the flare produced at each site was measured 5 min after completing irradiation. By 1 h the flare had resolved and at that time the intensity of the abnormal erythemal response, localized to the irradiation sites, was measured by reflectance spectrophotometry. The irradiation procedure and measurements were repeated during treatment with terfenadine (60–120 mg b.d.). Treatment with terfenadine significantly increased the smallest dose of light required to produce a flare (median increase 18 J/cm^2, $P = 0{\cdot}022$, Wilcoxon signed rank test; 95% confidence interval [12, 30 J/cm^2]) but there was no significant change in intensity of erythema localized to the irradiation sites.

Five patients were treated with β-carotene (30–90 mg daily). The flare and erythemal responses were unchanged after 2 months of treatment. However, the smallest dose of light required to produce a flare was again increased during additional treatment with terfenadine.

These results show that histamine release is one mechanism of photosensitivity in EPP and suggest that treatment with potent H_1 receptor antagonists may be of therapeutic value. β-carotene was not shown to provide protection using this testing procedure.

Cellular and cutaneous aspects of ciprofloxacin photosensitization. N.K.Gibbs, M.Henderson and B.E.Johnson, *Department of Dermatology, University of Dundee, U.K.*

We have reported preliminary studies of the phototoxic potential of ciprofloxacin (CIP), a broad-spectrum, quinolone antibiotic chemically related to nalidixic acid (NA) (Ferguson *et al.*, *J Invest Dermatol* 1988; **91:** 385). We set out to: (i) confirm CIP and NA phototoxicity in a standard Chinese hamster V79 cell line irradiated in microwells (UVA: glass filtered Philips BL 20W/08 fluorescent tubes); (ii) quantify the phototoxicity of CIP (i.p.) in mouse skin by ear-thickness measurements (UVA: acetate sheet filtered Sylvania PUVA) and to relate the photosensitivity to plasma levels of CIP (μg/ml), determined by a standard microbiological assay.

With V79 cells, both drugs (50 μg/ml in culture medium) produced a UVA dose (~0–112 kJ/m^2) related inhibition of mitochondrial dehydrogenase activity (MDA) and total cell protein synthesis (TPS), assayed *in situ* at 24 h and 4 days post-irradiation respectively (> 3 experiments/dose), with NA > > CIP. In

TABLE 1.

	Time (h)						
	0·25	0·5	1	1·5	2	3	4
Δ Thickness (SE)	0·09 (0·01)	0·1[illegible] (0·0[illegible])	0·10 (0·01)	0·11 (0·01)	0·07 (0·01)	0·01 (0·003)	0·01 (0·002)
Plasma (SE)	7·07 (0·3)	5·2[illegible] (1·3)	5·78 (0·7)	4·30 (0·6)	2·50 (0·3)	2·30 (0·3)	0·86 (0·1)

culture medium, pre-irradiated CIP induced some cell damage whilst pre-irradiated NA was highly toxic. The UVA doses (~kJ/m^2) for 50% inhibition of MDA were; CIP 31 (pre-irradiated 86), NA 8 (pre-irradiated 23) and for TPS were; CIP 18 (pre-irradiated 112), NA 6 (pre-irradiated 10).

A CIP concentration (0, 0·2 and 2·0 mg/mouse; 5 mice/concentration) dependent increased ear thickness (mm) was maximal 2 days after 5 days of repeated CIP photosensitization to UVA (~15 kJ/m^2). Maximum photosensitivity was between 0·25 and 1·5 h after injection (2 mg/mouse) with no effect after 3 h, which correlated approximately with CIP plasma levels (Table 1).

The V79 study confirms that CIP photosensitizes nucleated mammalian cells and suggests that CIP photoproducts may contribute minimally to this damage (this mechanism may be more important in NA photosensitization). These data, and the previous finding that CF has no photohaemolytic activity, indicate non-membrane, possibly nuclear, cellular targets. Our pharmacokinetic study suggests that in mouse skin the major chromophore for damage may be CIP itself or a metabolite with antibacterial activity.

Expression of endothelial leucocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) in the evolution of cutaneous inflammation: a comparison of ultraviolet-B (UVB) erythema and delayed type hypersensitivity (DTH).

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Leucocyte traffic in inflammation is likely to be controlled by the expression of cytokine-regulated cell surface adhesion molecules such as ELAM-1 and ICAM-1. ELAM-1 is present specifically on activated endothelial cells (EC) and serves as a receptor for neutrophils. ICAM-1 is present on unstimulated EC and activated mononuclear cells (MNC) and keratinocytes (KC), and serves as a receptor for lymphocytes and possibly neutrophils.

We have compared the expression of these molecules in skin biopsies from nine volunteers at different times up to 7 days following initiation of non-immune inflammation (two minimal erythema doses of 300 nm irradiation) or a DTH response to intradermal PPD, using monoclonal antibodies and an immunoperoxidase technique. In both groups, ELAM-1 was detected weakly on EC at 1 h and more strongly at 6 and 24 h. At 72 h ELAM-1 was still strongly expressed in the PPD group, but had decreased in the UVB group. By 7 days, ELAM-1 expression had dropped to low levels in both groups. ICAM-1 was strongly expressed on EC in unstimulated controls and in both groups at all timepoints. Differences were however readily observed in the expression of ICAM-1 on KC, which showed strong positive staining that appeared from 24 h to 7 days following PPD but which was absent at all time points following UVB. Perivascular MNC showed ICAM-1 staining in both groups but were considerably more numerous following PPD, particularly after 24 h.

These observations suggest that alterations in expression of ELAM-1 and ICAM-1 that occur up to 24 h may be attributable to non-specific inflammation, but that in DTH further alterations in adhesion molecule expression after 24 h may underlie the delayed nature of the MNC infiltration. Further studies will determine the role of lymphokines such as gamma interferon, known to induce KC ICAM-1 expression *in vitro*, in this process.

Evidence of a serological histamine-releasing factor in chronic urticaria. C.E.H.GRATTAN, *Department of Dermatology, The General Hospital, Birmingham, U.K.*

A serological mediator can be demonstrated in some patients with chronic urticaria by a weal and flare response on intradermal skin testing with autologous serum (Grattan CEH *et al.*, *Br J Dermatol* 1986; **114**; 583–90). The aim of this study was to look for involvement of histamine in the serum skin test response and to see whether pretreatment with a known mast-cell degranulating agent, compound 48/80, would modify the skin test response to serum.

All skin tests were performed by intradermal injection of 50 μl aliquots into volar forearm skin of chronic urticaria patients showing a response to intradermal autologous serum. Weal and flare areas were measured over 2 h in three patients' skin tested to autologous serum, normal saline (NS), 48/80 (10 μg) and histamine (1 μg). All other skin tests were measured as weal volumes at 60 min.

The serum skin test response followed a similar time course to histamine and 48/80 up to 1 h but persisted longer than histamine. Tenfold dilutions of serum in NS ($n=5$) reduced mean weal volumes by 61% at 1/10, 89% at 1/100 and 90% at 1/1000. Weal volumes were reduced by 79% with H1 blockade alone (terfenadine 120 mg b.d.) and by 80% with additional H2 blockade (cimetidine 400 mg q.d.s.) ($n=3$). Corresponding values for histamine (5 μg) were 90% and 84%. Tachyphylaxis of the serum skin response was almost complete (98%) over sites which had been repeatedly injected with 48/80 (100 μg) over the previous 24–48 h until there was no further response ($n=3$). Tachyphylaxis was also seen over skin pretreated with serum (99%) and histamine (77%) but not saline.

The results indicate that histamine is important in the chronic urticaria serum skin test response and may be released by mast cells in response to a serum factor or factors.

Cutaneous endothelial cell responses to histamine and substance P. HELEN A.BULL AND PAULINE M.DOWD, *Department of Dermatology, The Middlesex Hospital, London, U.K.*

Histamine is an important mediator of cutaneous inflammatory reactions, facilitating leucocyte margination, plasma extravasion and vasodilation. Its precise mode of action on cutaneous vascular endothelium is poorly understood, however it is thought to be mediated, in part, by release of prostaglandins. Furthermore, *in vivo* studies indicate that histamine may act synergistically with the vasoactive neuropeptide substance P (SP) in cutaneous inflammatory reactions. We have therefore sought to determine the mechanisms of action of histamine on dermal endothelial cells and whether these actions are modified by SP.

Dermal microvascular endothelial cells (DMEC) were cultured from human neonatal foreskin and confluent monolayers incubated with histamine (10^{-7}–10^{-3} M). Release of PGE_2 was concentration-dependent and DMEC incubated with 10^{-5} M histamine released $38{\cdot}4 \pm 9{\cdot}72$ pg/10^4 cells PGE_2 (mean ± standard error) ($n=4$). In comparison, interleukin 1 (1 U/ml) induced release of $147{\cdot}9 \pm 67{\cdot}6$ pg/10^4 cells PGE_2 ($n=2$).

Histamine-induced release of PGE_2 was inhibited by the H_1 antagonist pyrilamine but not by the H_2 antagonist cimetidine, nor was there an increase in intracellular c-AMP or c-GMP above baseline.

In separate experiments, DMEC were incubated with histamine (10^{-4} M) and SP (10^{-5} M), either alone or in combination (Table 1).

These results indicate that both histamine and SP release small amounts PGE_2 from DMEC. Histamine-release of PGE_2 is facilitated by H_1 receptor occupancy but not mediated by cyclic nucleotide second messengers. Furthermore, the effects of histamine and SP are additive and not synergistic.

TABLE 1.

	PGE$_2$ (pg/10^4 cells)	c-AMP (pg/500 μl)	c-GMP (pg/500 μl)
Histamine ($n = 3$)	13·94 ± 4·77	2·53 ± 1·08	0·49 ± 0·21
SP ($n = 3$)	21·63 ± 9·01	1·13 ± 0·43	0·82 ± 0·15
Histamine + SP ($n = 3$)	29·93 ± 10·42	4·23 ± 1·73	0·75 ± 0·45

Plasma-associated lymphocyte chemoattractant (PALC)—further characterization. K.B. Bacon, N.J.Fincham, A.I.Mallet and R.D.R.Camp, *Institute of Dermatology, St Thomas's Hospital, London, U.K.*

We have previously described the recovery of low molecular weight lymphocyte chemoattractant material from normal human stratum corneum, skin chamber fluid and plasma (PALC) (Bacon KB, Camp RDR, *J Invest Dermatol* 1989; **92:** 398). Further characterization of this material has now been undertaken.

Pooled PBS (4 ml) obtained from chambers applied to sellotape-stripped normal skin for 30 min, and normal plasma (300 ml), were purified by successive ultrafiltration and reversed-phase (RP) HPLC. Each source yielded a compound of high chromatographic purity with similar UV absorbance spectra ($\lambda_{max} = 270$–274 nm) and an identical retention time on isocratic RP-HPLC, chromatographing after admixture of equal amounts as a single peak at 8 min on a Brownlee C8 column, eluted with water/acetonitrile/trifluoroacetic acid (95:5:0·1, v/v). The purified material from plasma and skin caused concentration-related, *in vitro* lymphocyte chemoattractant activity (LCA) (Bacon KB *et al.*, *Br J Pharmacol* 1988; **95:** 966–74), with mean maximal migration indices of 2·0 and 2·3 respectively. Although the LCA was not extractable into ethyl acetate or chloroform/methanol from unpurified plasma YM10 filtrates, the purified plasma-derived compound (PALC) partitioned completely from water into these lipid solvents ($n = 3$ each). Incubation of PALC with high concentrations of polyacrylamide-bound trypsin (5 mg/ml) caused no loss of LCA on comparison with control incubations ($n = 2$), and amino acid analysis and microsequencing yielded only trace amounts of contaminating peptide material. Plasma desorption mass spectrometry of PALC indicated a molecular weight of 1183. PALC also chromatographed as a single, late-eluting, UV-absorbing, biologically active peak on TSK G2000SWXL gel filtration HPLC.

Normal plasma and skin chamber fluid therefore contain similar or identical, polar, low molecular weight, non-peptide, lipid-like lymphocyte chemoattractant material, which play a role in physiological lymphocyte trafficking. The full structure of this material remains to be elucidated.

Characterization of the cellular response to intradermal injections of IL-1α-like material derived from normal human stratum corneum. J.S.Ross, N.J.Fincham and R.D.R.Camp, *Institute of Dermatology, St Thomas's Hospital, London, U.K.*

As part of a study of the inflammatory properties of human interleukin (IL) 1 and in view of the lack of availability of clinical grade recombinant IL-1, we have now characterized the early cellular infiltrate seen in response to intradermal injections of autologous IL-1α-like material (60 U) which was purified from the heel stratum corneum of three volunteers by reversed-phase and anion-exchange HPLC. Erythema and swelling which were seen at IL-1 injection sites by 2 h and persisted for >24 h, were not due to contaminating endotoxin as injections of heat-treated material failed to elicit a response. Biopsies (4 h) of IL-1 and control injection sites were subjected to haematoxylin and eosin and special staining and examined by light microscopy. Results (mean numbers of cells per five high powered fields ± SEM, $n = 3$) are shown in Table 1.

The early response of autologous IL-1α-like material in human skin is therefore dominated by substantial increases in neutrophils and monocyte/macrophages, which may play a role in causing the early

TABLE 1.

	Control (PBS)	IL-1
Mononuclear cells (H and E)	302 ± 64	605 ± 7*
Neutrophils (chloracetate esterase)	1 ± 1	49 ± 15*
T-helpers (Leu 3a)	97 ± 8	167 ± 25**
T-suppressors (Leu 2a)	20 ± 3	25 ± 5
Monocyte/macrophages (OKM5)	94 ± 15	284 ± 42*
Monocyte/macrophages (Mac 387)	80 ± 11	351 ± 53*

* $P < 0{\cdot}05$ and ** $P = 0{\cdot}06$ in comparison to PBS (Student's *t*-test).

erythema and swelling seen. The mechanism of induction of these cellular infiltrates remains to be determined, as current evidence suggests that IL-1 is not directly chemotactic towards neutrophils and monocytes. Although IL-1 is directly chemotactic for T-helper cells, significant increases in these cells were not seen, but may occur during the later phase of the reaction.

Inhibition of interleukin (IL) 8-induced lymphocyte migration by calcium-channel antagonists. K.B.BACON, J.WESTWICK* AND R.D.R.CAMP, *Institute of Dermatology, St Thomas's Hospital and *Hunterian Institute, Royal College of Surgeons, Lincoln's Inn Fields, London, U.K.*

The migration of lymphocytes infiltrating inflammatory skin lesions may be induced by specific chemoattractants, whose mechanism of action may involve calcium-channel activation leading to altered intracellular calcium concentration. Since material similar or identical to IL-8 is found in lesional psoriatic skin and IL-8 is chemotactic for lymphocytes (Larsen CG *et al.*, *Science* 1989; **243:** 1464–6) the effects of calcium-channel antagonists on IL-8-induced lymphocyte migration have been investigated.

The effects of nifedipine (N), verapamil (V) and diltiazem (D; 10^{-10}–10^{-4} M in each case) and the inhibitory (−) and stimulatory (+) optical isomers of SDZ 202-791 (10^{-12}–10^{-6} M) on human recombinant IL-8-induced migration have been investigated by using a 48-well microchemotaxis assay, results being expressed as a migration index (Bacon KB *et al.*, *Br J Pharmacol* 1988; **95:** 966–74). Reversal of the antagonist effects of 10^{-8} M (−)-isomer by the (+)-isomer of SDZ 202-791 over the dose range (10^{-12}–10^{-4} M), was also attempted.

IL-8 (10^{-15}–10^{-10} M) induced concentration-related lymphocyte migration ($ED_{50} = 10^{-12}$ M; $n = 5$) which was abolished by addition of 3 mM EGTA to the assay medium ($n = 3$). Dose-related inhibition of lymphocyte migration in response to IL-8 (10^{-11} M) was obtained in the presence of either N, V or D, approximate IC_{50} values being 10^{-8} M, 6×10^{-8} M and 10^{-8} M, respectively ($n = 4$). The (+)-isomer of SDZ 202-791 was without effect on IL-8-induced migration but the (−)-isomer inhibited migration in a dose-related manner, the approximate IC_{50} value being 10^{-10} M ($n = 3$). In the presence of increasing concentrations of the (+)-isomer, the inhibitory effects of (−)-isomer on IL-8-induced migration were completely reversed, the approximate ED_{50} value being 10^{-10} M ($n = 3$).

The divalent cation-dependent induction of lymphocyte migration by IL-8 and its specific inhibition by calcium-channel blocking drugs, suggest that the therapeutic potential of these agents in lymphocyte-dependent inflammatory, autoimmune and allergic reactions, merits further investigation.

Inhibition of phospholipase A_2 in extracts of lesion-free psoriatic epidermis by the nonapeptide HDMNKVLDL which corresponds to lipocortin 1 residues 246-254. P.H.CARTWRIGHT, E.ILDERTON, J.M.SOWDEN AND H.J.YARDLEY*, *Department of Dermatology, North Staffordshire Hospital Centre, Stoke-on-Trent, and *Department of Biological Sciences, University of Keele*

Phospholipase A_2 (PLA_2) activity is raised in extracts of lesion-free psoriatic epidermis

(Forster S *et al.*, *Br J Dermatol* 1985; **112:** 135–47). PLA_2 releases free arachidonic acid from phospholipids present in cell membranes. Arachidonic acid is a precursor of both prostaglandins and leukotrienes which are potent mediators of inflammation.

The activity of PLA_2 is controlled by the protein lipocortin. We have previously shown that human recombinant lipocortin 1 inhibits epidermal PLA_2 activity in picomolar concentrations. Peptides corresponding with sequences found in lipocortin 1 strongly inhibit porcine pancreatic PLA_2 in concentrations of 5×10^{-8} M (Miele L *et al.*, *Nature* 1988; **335:** 726–30). We have studied the effect of the nonapeptide with the highest PLA_2 inhibitory activity in extracts of human psoriatic epidermis.

Superficial shave-skin biopsies were obtained under ethyl chloride local anaesthesia from the upper arm or back in six patients with chronic plaque psoriasis. Skin specimens were obtained at least 5 cm from the nearest lesion. Epidermal extracts were incubated with or without the nonapeptide HDMNKVLDL at 5×10^{-8} M for 15 min before measuring PLA_2 activity.

For untreated extracts the mean PLA_2 activity ± SEM was 125·5 ± 15·2 nmol/mg protein/h ($n = 6$). For extracts assayed after treatment with the nonapeptide, the PLA_2 activity was 65·9 ± 7·6 nmol/mg protein/h ($n = 6$). The difference is statistically significant ($P \simeq 0{\cdot}01$, paired t-test).

These results show that a synthetic nonapeptide related to lipocortin can significantly reduce the high PLA_2 activity in extracts of psoriatic epidermis. This and similar compounds offer exciting therapeutic possibilities for the future.

Purification of epidermal phospholipase A_2 and its inhibition by unsaturated fatty acids.

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The biosynthesis of pro-inflammatory eicosanoids via lipoxygenase and cyclo-oxygenase pathways is regulated by the availability of free arachidonic acid. This in turn is dependent on the activity of phospholipase A_2 (PLA_2) in releasing esterified arachidonic acid from cellular phospholipid stores (Irvine, *Biochem J* 1982; **204:** 2). Since PLA_2 has been reported to be abnormal in psoriasis (Forster *et al.*, *Br J Dermatol* 1983; **108:** 103) we have isolated and characterized epidermal PLA_2 in order to investigate its regulation.

Epidermal PLA_2 was purified from mouse-isolated epidermal cells by detergent solubilization, ion exchange chromatography and gel filtration. The enzyme was found to have a MW of 68 000, a pH optimum of 9·0 and an absolute dependence on Ca^{2+} ions for enzyme activity. Enzyme activity was detectable using both unilammelar vesicles and detergent micelles of phospholipid with no apparent preference for polar head group. PLA_2 activity was inhibited by unsaturated fatty acids (Ki 3–75 μM) in the following order of decreasing potency; ETYA > Arachidonate > Eicosapentaenoate > Linoleate = Linolenate > Oleate > Palmitate.

A similar PLA_2 with alkaline pH optimum and Ca^{2+}-dependence was present in human epidermal suction blisters. Human epidermal PLA_2 was also inhibited by arachidonic acid (Ki 5 μM) suggesting a regulatory role for feedback inhibition in the control of PLA_2 activity. Ki for arachidonic acid was found to be higher in uninvolved psoriatic epidermis (> 50 μM) than in control epidermis.

These results suggest that epidermal PLA_2 is regulated by product inhibition and indicate that this control mechanism may be defective in psoriasis.

POSTERS

The generation and effects of eicosanoids in the dermal equivalent model.

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The culture of dermal fibroblasts in a collagen matrix (dermal equivalent) is thought to be more representative of the *in vivo* situation than monolayer cultures. The generation and effects of eicosanoids in dermal equivalents have not been investigated.

Dermal equivalents were prepared containing 2×10^5 human dermal fibroblasts per 5 ml rat-tail collagen gel (3 mg/ml) in Dulbecco's MEM containing fetal calf serum (FCS) plated in 5-cm dishes. Gel contraction was measured as the reduction in the mean of two perpendicular diameters. PGE_2 was measured by RIA.

Dermal equivalent contraction was less, $35 \pm 3\%$, in 1% FCS compared with $48 \pm 3\%$, ($P < 0{\cdot}02$, $n = 8$) for 10% FCS. Arachidonic acid, PGE_2, 5 (RS)-HETE, 12 (RS)-HETE and 15 (RS)-HETE (10^{-5}–10^{-9} M) tended to further inhibit contraction although the reduction was small and not consistently significant. In no instance was the contraction rate increased.

PGE_2 synthesis was highest during the initial 48 h of culture, when gels were rapidly contracting, and subsequently declined to minimal levels. Initial release was significantly higher for gels in 10% FCS (26 ± 5 ng/culture per 48 h, $n = 6$) compared with 1% FCS ($3 \pm 0{\cdot}7$ ng/culture). Addition of TGF-β (0·1 to 10 ng/ml) neither affected gel contraction nor PGE_2 release.

These results suggest that there is no direct relationship between eicosanoid synthesis and the interaction between fibroblasts and the collagen fibrils. It appears unlikely that eicosanoids play a part in collagen remodelling in wound healing. In contrast to a previous report we were unable to demonstrate an effect of TGF-β on gel contraction.

Blood polymorphonuclear cells from patients with psoriasis show an increased adherence to cultured vascular endothelium. K.A.BROWN, F.LEROY, M.W.GREAVES* AND D.C.DUMONDE, *Department of Immunology and *Institute of Dermatology, UMDS, St Thomas's Hospital Campus, London U.K.*

The infiltration of blood polymorphonuclear cells (PMNs) into the epidermis of psoriatic skin to form microabscesses is probably initiated by the increased attachment of these cells to local vascular endothelium. Cytokines, such as IL-1, are present in psoriatic lesions (Camp RDR *et al., J Immunol* 1986; **137:** 3469) and these are known to act on cultured endothelium to increase their adhesiveness for PMNs (Bevilacqua MP *et al., J Clin Invest,* 1985; **76:** 2003). The aim of this study was to determine if blood PMNs from patients with psoriasis were more adherent than control PMNs to untreated and cytokine-treated cultured human endothelium.

Fourteen psoriatic patients and 14 controls were studied. Adherence was quantitated by measuring the binding of ^{51}Cr-labelled PMNs to confluent monolayers of human umbilical vein endothelium grown in microwells. PMNs, suspended in 10% autologous serum and RPMI medium, were incubated with endothelium for 1 h at 37°C. Prior to the adherence assay, endothelial monolayers were pretreated for 4 h with 0·02–2·0 units/well of recombinant IL-1 or tumour-necrosis factor (TNFα). Each test was performed in quadruplicate.

Psoriatic PMNs were more adherent than control PMNs to untreated endothelium (mean 35% increase; $P < 0{\cdot}05$). There was no difference in the responsiveness of PMNs from both groups of subjects to cytokine-treated endothelial cells. PMNs from controls and patients exhibited a mean 59% and 48% increase respectively ($P < 0{\cdot}01$) to IL-1-treated endothelial cells and a 112% and 103% increase ($P < 0{\cdot}001$) to endothelial cells pretreated with TNFα. We propose that blood PMNs from patients with psoriasis may already be predisposed to an increased extravasation into skin lesions.

Expression of mRNA recognized by two pigment-cell-specific cDNA clones and tyrosinase synthesis during the hair growth cycle. S.A.BURCHILL, C.P.F.REDFERN AND A.J.THODY, *Dermatology Department, University of Newcastle upon Tyne, U.K.*

pMT5 and pmc Tyr-B9 are pigment-cell-specific cDNA clones which have been claimed to encode mouse tyrosinase (Shibahara A *et al., Nucl Acid Res,* 1986; **14:** 2413–27; Muller G *et al., Embo J,* 1988; **7:** 2723–30). However, recent evidence suggests that pMT5 may code for a tyrosinase-related-protein (TRP). In this study we have examined the expression of mRNA recognized by both these cDNAs and their relationship with tyrosinase synthesis during hair growth in the mouse.

Skin samples for RNA extraction and measurement of tyrosinase synthesis were taken 0–16 days after birth during the first hair cycle. Northern blots of poly(A)+RNA were probed with [^{32}P]-labelled cDNA inserts of pMT5 or pmc Tyr-B9 and subsequently reprobed for β-actin to check the loading of poly(A)+RNA. Tyrosinase synthesis was measured by immunoprecipitation of [^{35}S]-methionine-labelled follicles with tyrosinase antibody.

Tyrosinase synthesis occurred within 4 days of birth, during the early anagen phase of the hair cycle. Synthesis peaked about 7–8 days after birth and then continued at a reduced level until late anagen. No synthesis occurred during catagen and telogen. During the anagen phase of hair growth mRNA was recognized by the pmc Tyr-B9 probe, and its expression was greatest 2–4 days after birth. No expression was found during catagen or telogen. However, the pMT5 probe recognized no mRNA during this first hair cycle.

The data demonstrate that the expression of mRNA recognized by the pMT5 probe and tyrosinase synthesis do not always correlate. This suggests that the pMT5 cDNA clone is coding for a protein distinct from tyrosinase. However, the increased expression of mRNA recognized by pmc Tyr-B9 during periods of high tyrosinase synthesis is consistent with the idea that this cDNA clone may code for mouse tyrosinase.

Is prognosis related to HLA-DR expression by malignant melanoma cells?

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HLA-DR, a class II major histocompatibility complex (MHC) antigen, is not expressed on the surface of benign melanocytic (naevus) cells. It is however expressed by malignant cells both in culture and '*in vivo*'. The presence of staining, the proportion of positive cells and their staining intensity varies depending on the technique used. No consensus on the significance of HLA-DR antigen expression has emerged, although a recent paper has proposed that positive expression is associated with a poor prognosis in invasive malignant melanoma.

The monoclonal antibodies LN3 and TAL 1B5, which are directed against α-chain HLA-DR epitopes, work on routinely processed and fixed tissues and are visualized using an avidin–biotin–alkaline phosphatase technique. We have applied them retrospectively to 35 cases of invasive primary cutaneous malignant melanoma with 5-year clinical follow-up data. Of these, 29 have a significant potential for metastasis using Breslaw's original criteria.

Fifteen out of 29 (52%) are HLA-DR positive, although the proportion of positive cells varies considerably from $<5\%$ to $>50\%$. Of these 15 cases, 5/15 (33%) have suffered either local recurrences or distant metastasis and 2/15 (13%) have died of disseminated malignancy. Fourteen out of 29 (48%) do not express detectable amounts of HLA-DR antigen. Of these, 11/14 (79%) have suffered local or distant metastasis, and 8/14 (57%) have died of disseminated malignancy. Local or distant metastasis ($P < 0{\cdot}02$) and mortality from disseminated malignant melanoma ($P < 0{\cdot}05$) are significantly correlated with failure to express HLA-DR antigen.

This preliminary study suggests that the expression of HLA-DR is a favourable prognostic factor which could usefully be included in the assessment of invasive melanocytic malignancies.

Nicotinamide, an *in vitro* and *in vivo* mononuclear cell cAMP phosphodiesterase inhibitor—its use in atopic dermatitis.

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Adults with atopic dermatitis (AD) have elevated activity of peripheral blood mononuclear cell (MNL) cAMP-specific phosphodiesterase (PDE). Nicotinamide (Nic) inhibits PDE in rat liver and reduces the severity and mortality of anaphylactic reactions in mice. We sought to determine if Nic-inhibited PDE in human MNLs *in vitro* and the effect of oral nicotinamide on MNL PDE and clinical parameters in patients with AD.

Previously frozen MNL homogenates from two normal subjects were assayed for PDE activity by radioenzyme assay in the presence of Nic varying in concentration between 2·5 and 120 mM Nic in a series of four experiments. A dose-related reduction in PDE activity was documented with 50% reduction occurring at a 15 mM concentration of Nic, being maximal (90%) at 60 mM. Enzyme kinetic studies suggested that inhibition was competitive. Using fresh unfrozen cells from eight normal subjects, only a 30% maximal reduction in PDE activity was produced with 60 mM Nic.

In an open study, 2 g oral nicotinamide was administered daily for 4 weeks to seven adults with atopic dermatitis. MNL PDE fell from 4·16 ± 0·72 to 2·95 ± 0·75 pmol/min per mg protein (mean ± SEM, $P < 0{\cdot}05$). There was no significant change in eczema extent, itch, or weal volumes produced by intradermal injection of 100 nmol histamine, 100 pmol Substance P and 25 units of house dust mite antigen.

Phenotypic characteristics of antigen-bearing cells in the draining lymph nodes of contact sensitized mice. M.CUMBERBATCH AND I.KIMBER, *ICI Central Toxicology Laboratory, Alderley Park, Macclesfield, U.K.*

Following the topical exposure of mice to contact-sensitizing chemicals there is a rapid accumulation of dendritic cells in the draining lymph nodes. Studies with the skin-sensitizing fluorochromes, fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) have revealed that within hours of skin painting there is the appearance of large antigen-bearing cells within lymph nodes. Such cells are immunogenic and efficiently transfer specific sensitization to naïve recipients. Although it has been presumed that antigen-bearing cells are dendritic in nature and derived from the Langerhans cells of the skin, the possibility remains that the recently discovered murine epidermal Thy 1^+ dendritic cell may transport antigen or that resident lymph node macrophages may bind antigen. To more accurately determine the phenotypic characteristics of antigen-bearing cells which accumulate in lymph nodes, we have performed two-colour immunofluorescence analyses with a panel of monoclonal antibodies. These studies have demonstrated that antigen-bearing lymph node cells express high levels of membrane Ia but lack Thy 1 and determinants associated with macrophages. Furthermore, unlike macrophages, antigen-bearing cells fail to phagocytose latex beads, even under optimal culture conditions.

The conclusion drawn is that cell-associated antigen within the draining lymph nodes of contact sensitized mice is found almost exclusively within the Ia^+, Thy-1^- dendritic-cell fraction.

Cyclic AMP-specific phosphodiesterase in atopic dermatitis—activity in whole skin and cultured fibroblasts. S.N.DUNCAN, I.H.COULSON, H.NAVSARIA,* I.LEIGH AND C.A.HOLDEN, *Skin Laboratory, St Helier Hospital, Carshalton, Surrey and *Experimental Dermatology Laboratory, The London Hospital, U.K.*

Cyclic AMP-specific phosphodiesterase (PDE) activity is significantly elevated in peripheral blood mononuclear cells in atopic adults, particularly those with atopic dermatitis (AD). Elevation of PDE activity in cultured keratinocytes derived from lesional atopic dermatitis skin has not been found. This would suggest that elevated PDE activity in AD is confined to bone marrow-derived immune-competent cells. To further investigate this, we sought to compare PDE activity in cultured fibroblasts and sonicated whole-skin derived from lesions of atopic dermatitis to skin derived from non-atopic controls.

Punch biopsies (4 mm) were taken from areas of active atopic dermatitis. Normal skin adjacent to pigmented lesions being removed for cosmetic reasons was collected from non-atopic individuals. Skin biopsies were cultured in medium 199 in a 5% CO_2, 95% humidity atmosphere at 37°C until confluence

TABLE 1.

	PDE activity (pmol/min per mg protein mean ± SEM)	
	Control	AD
Cultured fibroblasts	1·39 ± 0·37 $n = 7$	0·79 ± 0·25 $P = 0·2$ $n = 7$
Sonicated whole skin	0·37 ± 0·1 $n = 15$	0·78 ± 0·61 $P = 0·5$ $n = 4$

was achieved at P_2 or P_3. Cultured fibroblast and whole skin PDE was determined on sonicates; preliminary experiments showed that optimal PDE activity was achieved by ultrasonicating whole skin for 3 × 20 s in 2 ml of GBSS. PDE activity was measured by radioenzyme assay, activity being related to total protein concentration (Table 1).

Significant differences in fibroblast and whole skin PDE between AD lesional skin and normal skin were not found. Elevated PDE in AD would seem to be confined to marrow-derived mononuclear cells.

The effect of oral antibiotic therapy for acne on the incidence of multiply-resistant coagulase-negative staphylococci at the skin surface.

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Multiply resistant (mult^R) coagulase-negative staphylococci (CONS) are increasingly being implicated in infections of compromised hosts. Cutaneous CONS rapidly develop resistance to the administered antibiotic during oral therapy for acne vulgaris. In addition non-responding antibiotic-treated acne patients have been shown to carry more mult^R CONS than untreated subjects. In this study the incidence of singly and multiply-resistant CONS before, during and after a standard 6-month course of oral tetracycline (500 mg b.d., 12 patients), minocycline (100 mg b.d., 13 patients) or erythromycin (500 mg b.d., 10 patients) was determined in 35 previously untreated acne patients. Collection of bacterial samples and detection of resistance were by standard methods. Before treatment, the following incidences (expressed as % of patients) of individual resistances were detected: penicillin 97, tetracycline 91·4, erythromycin 57, fusidic acid 57, trimethoprim 31·4, chloramphenicol 23, clindamycin 5·7, gentamicin 5·7. The number of resistant strains cm^{-2} skin varied by > 5 log cycles between patients, whereas the proportion of resistant strains varied from $< 10^{-3}$ to 100% of the total staphylococcal flora of individual patients. Mult^R staphylococci were carried by 51·4% of patients before treatment and 28·6% carried > 100 such strains cm^{-2} skin. After 1 week of treatment none of the three antibiotics produced a significant increase in the number of mult^R staphylococci. Increasing duration of therapy was associated with a rise in the number of mult^R strains irrespective of the antibiotic used. After 6 months, the number of patients carrying > 100 mult^R staphylococci cm^{-2} skin had risen to 68·6% ($P < 0·01$, χ^2). Overall, minocycline treatment resulted in less multiple resistance than tetracycline or erythromycin. The results show that the large pre-existing reservoir of mult^R CONS on the skin of untreated subjects is significantly increased following long-term oral administration of antibiotics for acne.

Retinoic acid-induced inhibition of metastatic melanoma cell-cell aggregation, adhesion to endothelium, and lung colonization.

J.GOLD, M.EDWARD AND R.M.MACKIE, *Department of Dermatology, University of Glasgow, U.K.*

Retinoids exert a wide range of biological effects on normal and tumour cells in culture and *in vivo*, the most important being the modulation of cell growth and differentiation. The mechanism(s) whereby retinoic acid inhibits melanoma cell lung colonization (demonstrated here) is not understood, but may, in part, involve inhibition of degradative enzymes such as collagenases, or the modulation of tumour cell adhesion. We have previously demonstrated retinoic acid-induced inhibition of melanoma cell adhesion to type IV collagen and laminin/nidogen, and now present evidence that retinoic acid pretreated melanoma cells exhibit reduced homotypic and heterotypic cell-cell adhesion.

B16F1M2 cells were derived from the B16F1 melanoma cell line by passage through the lungs of C57BL mice twice, producing a highly metastatic cell line. Lung colonization potential was assessed by injecting 10^5 cells via the tail vein of C57BL mice and counting lung nodules 18 days later. For cell-adhesion studies B16F1M2 cells were grown in the presence or absence of 10^{-6} M retinoic acid for 4 days and labelled with ^{51}Cr-chromate for 18 h prior to detachment. Washed cells were seeded onto confluent monolayers of either B16F1M2 melanoma cells or calf pulmonary aortic endothelial cells, in serum-free medium, and incubated at 37°C. At various time intervals cell layers were washed with PBS, and adherent cells solubilized with 0·5 M NaOH and aliquots taken for gamma counting. Spheroid formation was examined by seeding cells on top of 0·5% agar.

Retinoic acid pretreatment of B16F1M2 melanoma cells reduced their adhesion to both melanoma and endothelial cell monolayers and also inhibited their ability to form spheroids. Inhibition of lung colonization may therefore be due, in part, to a reduction in the formation of tumour-cell aggregates which may non-specifically lodge in microcapillaries, or reduced adhesion of the tumour cells to the endothelium of the target organ.

Demonstration of the systemic effect of topical treatment in psoriasis by clinical assessment and blood-flow measurement.

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We wished to investigate the effect of generalized topical treatment on a single untreated plaque of psoriasis, and used clinical assessment and laser-Doppler flowmetry to do this.

Ten patients with chronic plaque psoriasis were assessed according to the PASI scoring system, and then a single plaque was chosen for more specific measurement. Erythema, scaling and induration were graded, blood flow was measured using a laser-Doppler flowmeter (Perimed, Pfld) at the centre of the plaque, the active edge and the inactive edge. Patients received conventional dithranol treatment, avoiding the plaque specifically assessed. The assessment was repeated after 1 week. Two patients also received UVB therapy, but the untreated plaque was covered during UVB exposure.

There was improvement in the psoriasis in nine out of 10 patients, with a fall in mean PASI scores from 8·6 to 4·2. The untreated plaque also improved, with a mean change in score of 3(2–5) (mean(range)). The blood flow at the centre of the plaque fell from 4·2 V (3·5–4·5) to 2·3 V (1·5–3), and at the active edge from 2·7 (1·5–4) to 1·6 (0·5–2·5). UVB treatment made no difference to the results. One patient's psoriasis worsened, and PASI scores, as well as blood flow, increased.

Therefore, there is clear evidence, both from clinical assessment and from objective blood flow measurement, of a systemic effect of topical treatment. This is true of dithranol alone, and combined with UVB. Dithranol is poorly absorbed through the skin, and it is unlikely that this is an important factor. It is more likely that changes in circulating factors, either humoral or cellular, are responsible. This has important implications both for the pathogenesis of psoriasis, and for the planning of therapeutic trials in this disease.

Effect of *N,N*-dimethylformamide on metastatic B16 melanoma cell adhesion, melanogenesis, glycosaminoglycan synthesis, and lung colonization.

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Polar solvents such as dimethylformamide (DMF) have been shown to induce tumour cell differentiation *in vitro* and *in vivo*, and may prove to be important in the treatment of certain tumours. Here we have examined the effect of DMF on murine metastatic melanoma cell growth, morphology, melanogenesis, adhesion to various substrates, glycosaminoglycan properties and ability to colonize the lungs of syngeneic C57 BL mice.

Cells were grown in the presence or absence of 0·5% DMF in 60-mm Petri dishes and counted daily. An aliquot of the cell suspension was taken for tyrosinase activity estimation while the remainder was used for melanin determination. For adhesion studies, cells were grown for 4 days in 0·5% DMF and seeded onto 35-mm Petri dishes coated with various proteins. Adherent cells were counted at various time intervals following incubation in serum-free medium. GAGS from the medium and cell surface fractions were examined on ion-exchange columns, while lung colonization was assessed by injection of 10^5 cells into the tail vein and lung nodules counted 18 days later.

DMF inhibited the growth of the melanoma cells, cell numbers being reduced by 25% following 4 days incubation in the presence of 0·5% DMF. Melanin synthesis was increased from 2·2 to 4·2 μg/10^5 cells, and tyrosinase activity increased twofold following 4 days incubation. DMF altered the properties of GAGS and reduced the adhesion of the melanoma cells to laminin/nidogen, but had little effect on adhesion to type I collagen and fibronectin, while the tumour cells' ability to colonize the lungs was increased, the average tumour count rising from an average of 75 to 105. While DMF appears to induce a more highly differentiated state it increased the cells' ability to colonize the lung. This may be due to possible changes in enzyme activities such as plasminogen activators, collagenases and heparanase.

Changes in epidermal keratin levels during treatment of psoriasis with the topical vitamin D_3 analogue MC903.

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In psoriasis the keratinocytes follow an alternate maturation pathway in which they hyperproliferate and fail to complete terminal differentiation leading to a modified keratin profile. Keratins 1 and 2 (markers of differentiation) are reduced and keratins 6 and 16 (markers of hyperproliferation) appear or are increased. Treatment of psoriasis with vitamin D_3 metabolites or analogues, are clinically effective and free from side-effects. We investigated the clinical efficacy of the vitamin D_3 analogue MC903 applied topically twice daily in eight patients with plaque psoriasis. The effects of MC903 on serum calcium, inorganic phosphate and 1,25 dihydroxyvitamin D_3 [1,25(OH)$_2$D$_3$] and on epidermal differentiation, as measured by changes in epidermal lesional keratins, were examined. Keratotome shavings and blood samples were taken before treatment and at 2–3 weekly intervals during therapy. Keratins were analysed by SDS–PAGE and quantified by scanning densitometry. Serum levels of 1,25(OH)$_2$D$_3$ were measured using an Amersham Kit. Clinical resolution occurred rapidly within 6–10 weeks, with no side-effects, in six patients. Serum calcium and phosphate levels remained within the normal range, while serum 1,25(OH)$_2$D$_3$ levels did not alter significantly throughout therapy. No correlation between 1,25(OH)$_2$D$_3$ levels and patient responsiveness to MC903 was found. The most consistent keratin changes during resolution of a lesion were a reduction in the amounts of keratins 16 and 18 and an increase in keratins 1 and 2. These changes were similar to those observed with oral 1α(OH)vitamin D_3 or with PUVA therapy. The decrease in the levels of K16 suggests that the treatment causes inhibition of keratinocyte proliferation, while the stimulation of the production of K1 and K2, suggests that a return to normal epidermal keratinocyte maturation occurs.

Expression and cytoskeletal binding of calgranulin in cultured keratinocytes.

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The molecules calgranulins A and B are two intracellular calcium-binding proteins which are expressed by the lesional keratinocytes of inflammatory dermatoses. We have investigated the induction of the calgranulin proteins in an *in vitro* system and characterized the epidermal form of calgranulin. Using calgranulin-specific monoclonal antibodies we have shown that these proteins are expressed within the epidermis of skin explants after 12–24 h culture *in vitro*. The induction of calgranulin-specific staining on culture was prevented, however, by the inclusion of cycloheximide in the culture medium, in sufficient quantities to prevent *de novo* mRNA synthesis. Indirect immunofluorescence staining was used to analyse the subcellular localization of the calgranulin proteins. The specific staining pattern with antibodies which recognize the individual calgranulin proteins was retained in detergent-insoluble cytoskeletal preparations in epidermal sections. The calgranulin-positive explants also displayed evidence of a cytoskeletal-filament redistribution typical of keratinocytes cultured under low-calcium conditions. Calgranulins were associated with these redistributed filaments.

Epidermal calgranulins were solubilized using a urea-based protein-extraction buffer. After sodium dodecyl sulphate (SDS)-polyacrylamide-gel electrophoresis a single antigen, with a molecular weight of 13·0 kDa was detected with the calgranulin specific antibody MAC 387. Extraction of the proteins from psoriatic scale also displayed an antigen at this molecular weight. This value agrees with that previously described for the calgranulin B sequence found in myelomonocytic blood cells.

The expression of calgranulins, similar to other members of the same protein family, may regulate cytoskeletal changes in skin disease.

Non-secretion of ABO blood-group antigens in patients with palmoplantar pustulosis without arthro-osteitis. D.KEMMETT, R.L.SLOAN,* C.C.BLACKWELL,† D.M.WEIR† AND G.NUKI*, *Departments of Dermatology, *Rheumatology and †Bacteriology, University of Edinburgh, U.K.*

The non-secretion of ABO blood-group antigens is associated with a number of bacterial and fungal infections and with some autoimmune diseases for which infectious triggers have been proposed. Non-secretion has also been found to be a host-susceptibility factor for ankylosing spondylitis (AS), reactive arthritis and psoriatic arthropathy (PsA) but not for rheumatoid arthritis or uncomplicated psoriasis (PS). The relationship of palmoplantar pustulosis with psoriasis remains controversial. There are clinical differences and the HLA associations of the diseases are distinct (Kemmett D *et al.*, *Br J Dermatol* 1985; **113:** 40). Recently, a characteristic form of arthro-osteitis has been found to be associated with PPP (Sonazaki H *et al.*, *Ann Rheum Dis* 1981; **40:** 554–7).

The secretor status of 36 patients with PPP was assessed. Patients with coexisting psoriasis, a family history of psoriasis or diabetes were excluded. None had clinical, radiographic or scintigraphic evidence of arthro-osteitis. The proportion of non-secretors (50%) was comparable to that found amongst 84 patients with AS (49%) and 42 patients with PsA (45%) and significantly higher than that found in patients with uncomplicated psoriasis (24%) or controls (27%) ($P < 0{\cdot}01$).

PPP can be added to the list of diseases associated with non-secretion of blood group antigens. Secretor status provides further evidence that PPP and PS are distinct entities.

The effect of griseofulvin on serum cholesterol. B.J.LIDDLE, J.R.MARSDEN AND R.J.CRAMB*, *Department of Dermatology, The General Hospital, Birmingham and *Clinical Chemistry, The Queen Elizabeth Hospital, Birmingham, U.K.*

Drugs which induce hepatic mixed function oxidases (MFO) such as phenytoin and phenobarbitone increase the concentration of serum cholesterol. Griseofulvin is reputed to induce hepatic MFO but the evidence for this is indirect (Okino K *et al.*, *Drug Intel Clin Pharm* 1986; **20:** 291–3). Therefore we have investigated the effect of griseofulvin on concentrations of α-1-acid glycoprotein (AAG) and gamma glutaryl transferase (GGT) which reflect MFO activity, and on levels of serum lipids and apolipoproteins. Ten patients with dermatophytosis (three females and seven males, aged 18–65) were treated with griseofulvin (10 mg/kg per day) for 8 weeks. Blood was taken after a 12-h fast before and at 2-weekly intervals during treatment, and at 2 and 4 weeks after treatment was stopped. All samples were analysed for: AAG, GGT, total serum cholesterol (C), triglycerides (T), apolipoprotein B (apo B), high density lipoprotein cholesterol (HDL C) and apolipoprotein A (apo A). Concentrations of AAG increased from

0·76 g/l ± 0·08 (SEM) to 1·15 g/l ± 0·21 (P 0·05) after 8 weeks treatment, and GGT levels were 25 U/l ± 0·24 pretreatment and 36 U/l ± 0·21 (P 0·05) after 8 weeks. C levels were 5·3 mmol/l ± 0·3 before griseofulvin and 5·4 mol/l ± 0·3 after 8 weeks treatment and T levels were 1·7 mmol/l ± 0·3 before and 1·6 mmol/l ± 0·3 at the end of treatment. Apo B levels were 0·76 mmol/l ± 0·05 before griseofulvin and 0·76 mmol/l ± 0·05 after 8 weeks treatment. Concentrations of HDL C were 1·1 mmol/l ± 0·1 before griseofulvin and 1·2 mmol/l ± 0·1 after 8 weeks, and apo A levels were 1·2 mmol/l ± 0·1 before griseofulvin and 1·3 mmol/l ± 0·1 after 8 weeks treatment. These findings suggest that griseofulvin induces hepatic MFO but that treatment for 8 weeks has no effect on concentrations of serum lipids or apolipoproteins.

The role of a basement-membrane-degrading collagenase in scleroderma. B.McLaughlin and J.B.Weiss, *The Wolfson Angiogenesis Unit, University of Manchester and Department of Rheumatology, Clinical Sciences Building, Hope Hospital, Salford, U.K.*

Skin fibroblasts in culture are known to secrete three neutral metalloendoproteinases: collagenase, stromelysin and gelatinase. Recently, it has been shown that gelatinase which was always thought to be an enzyme of rather wide specificity is in fact a specific basement membrane (type IV collagen) degrading enzyme (Collier *et al.*, *J Biochem* 1988; **263:** 6579–87).

In scleroderma the normal response to vascular damage is not observed and we examined the possibility that the development of the peripheral microvascular pathology might be due to an abnormal, possibly transformed, fibroblast producing a basement-membrane-degrading enzyme which would inhibit angiogenesis and subsequent repair. We have examined nine scleroderma skin fibroblast cell lines and observed that five of them secreted a significantly increased amount of latent gelatinase as compared with age-and sex-matched control skin fibroblasts from normal skin.

The average amount of latent gelatinase secreted by the five cell lines expressed as ng of gelatin degraded per hour was 2380 ± 510. This compared with 273 ng degraded per h ± 41 for the controls. This gives a significance of > 0·05. In these experiments the latent enzyme was activated with ESAF.

It is possible that in normal circumstances vascular damage and subsequent local ischaemia would be followed by vascular proliferation (angiogenesis) which would either return the vascular bed to normal or possibly result in haemorrhage and overproliferation of microvessels (e.g. retinopathy). However, in scleroderma increased levels of type IV collagenase would degrade the basement membrane and therefore interfere with the formation of capillaries and other more major blood vessels. Ischaemic cells generally respond by producing ESAF and this would result in activation of latent enzyme and result in the lysis of the basement membrane and the production of highly unfavourable environments for new capillary growth.

The effect of treatment with 13-*cis* retinoic acid on the metabolic burst of peripheral blood neutrophils from patients with acne. W.Perkins, E.V.Crocket,* M.B.Hodgins, R.M.MacKie and J.M.Lackie*, *Department of Dermatology and *Department of Cell Biology, University of Glasgow, U.K.*

Neutrophils are implicated as mediators of tissue damage in inflammatory disease. It has been suggested that the therapeutic benefit of 13-*cis* retinoic acid (13-*cis*) in acne may result in part from effects on neutrophil function. The metabolic burst in response to pro-inflammatory stimuli is important in mediating tissue damage by neutrophils and can be quantified by luminol-enhanced chemiluminescence assay (Lackie JM, Lawrence AJ, *Biochem Pharmacol* 1987; **36:** 1941–5). In the present study, the formyl peptide-induced metabolic burst was studied in peripheral blood neutrophils of acne patients undergoing treatment with oral 13-*cis* at a dose of 1·0 mg/kg per day. Neutrophils were isolated from venous blood by Ficoll–Hypaque density gradient centrifugation, stimulated *in vitro* with formyl peptide (f-met-leu-phe, 100 nmol/l) and assayed for chemiluminescence (units/10^6 cells). The mean chemiluminescence response was significantly greater ($P < 0·05$) in patients receiving 13-*cis* (113 + 13 SEM, $n = 32$) than in untreated patients (71 + 14 SEM, $n = 21$) or age-matched non-acne controls (55 + 5 SEM, $n = 24$). Pre-incubation of neutrophils *in vitro* with 13-*cis* (10 nmol/l) did not affect the chemiluminescence response to formyl peptide. A sequential study over 20 weeks in seven patients showed that chemiluminescence peaked between 2 and 8 weeks of treatment. In three patients this was accompanied by worsening of their acne.

These studies suggest that, in the initial phase, treatment with 13-*cis* might exacerbate certain neutrophil-mediated inflammatory processes in acne.

Failure of psoriatic fibroblasts to promote keratinocyte proliferation *in vitro*.

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The various abnormalities of fibroblasts cultured from psoriatic dermis could indicate a primary role for fibroblasts in inducing epidermal hyperplasia, as proposed by Saiag *et al.* (*Science* 1985; **230:** 669–72), or that events in both dermis and epidermis are triggered by an extracutaneous stimulus, for example, by activated T lymphocytes (Valdimarsson *et al*, *Immunol Today* 1986; **7:** 256–9). We examined the first possibility.

Keratinocytes from normal infant foreskins, cultured without feeder layers, received medium conditioned by normal (HSF) or psoriatic (PSA) fibroblasts or medium pre-incubated without cells. In experiments with six different pairs of fibroblast lines the mean increases (± SEM) in proliferation from cultures fed the unconditioned medium were 16 ± 4% (HSF) and 6 ± 7% (PSA) ($P>0{\cdot}05$). In two other experiments, fibroblasts in collagen lattices were co-cultured with keratinocyte monolayers in multi-well dishes using cellulose acetate inserts. Although HSF-populated lattices had no effect, PSA-populated lattices depressed proliferation by 35 ± 5% and 30 ± 8% ($P<0{\cdot}01$) compared to lattices without fibroblasts. As a positive control, keratinocyte cultures were also fed leucocyte culture supernatants and proliferation increased by up to 65%. These findings do not support an inductive role for dermal fibroblasts in psoriasis.

Determination of the responsiveness of lymphocyte subsets to locomotor stimuli by immunocytochemical analysis of migrated cells.

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We have previously reported the concentration-related *in vitro* migration of mixed peripheral blood lymphocytes (PBL) in response to recombinant (r) interleukin (IL) 1α (0·25–5 ng/ml) and zymosan activated plasma (ZAP; 0·1–5% v/v). Others have attempted to determine the responsiveness of lymphocyte subpopulations to chemotactic stimuli by examining the migration of specifically purified PBL subsets. We have now studied the migration of mixed PBL, purified by density gradient centrifugation and plastic adherence, in a microchemotaxis assay in the presence of minimal essential medium (MEM) alone and in response to rIL-1α (5 ng/ml) and ZAP (5% v/v). APAAP immunochemistry and the monoclonal antibodies Royal Free Pan-T, Royal Free Pan-B, Dako-T4 and Dako-T8 were used to

TABLE 1.

	IL-1	MEM	*n*	ZAP	MEM	*n*
Pan T	181 ± 26*	84 ± 13	4	168 ± 24*	84 ± 11	5
Pan B	12 ± 3	7 ± 3	4	24 ± 4†	11 ± 4	6
T-helper	164 ± 19*	72 ± 3	3	56 ± 10*	28 ± 3	5
T-suppressor	37 ± 6	35 ± 10	4	72 ± 12*	33 ± 6	6

* $P<0{\cdot}05$ and † $P=0{\cdot}05$ on comparison with MEM alone (paired *t*-test)

respectively quantify total T cells, total B cells, T-helpers and T-suppressors, which had migrated to the undersurface of polycarbonate chemotaxis filters. Results (mean numbers of cells per eight high power fields ± SEM) are shown in Table 1.

These results show that the majority of migrating PBL are T-cells. rIL-1α selectively stimulated the migration of T-helper cells without affecting T-suppressor locomotion. ZAP significantly stimulated the migration of both T-helper and T-suppressor cells and B cells. This method may be used to investigate the selective actions of lymphocyte chemoattractants and to study the specificity of the effects of certain drugs on PBL migration.

The effects of species and density of infection on the pathology of ringworm.

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The relationships between the quality and quantity of a noxious stimulus and the ensuing tissue response are important to an understanding of the pathodynamics of all disease. In human ringworm infections the fungal micro-organisms are confined to the stratum corneum, but the inflammatory reaction occurs in the viable tissue beneath, and to establish stimulus-response relationships both anatomical zones must be studied.

In this study 18 patients (18–60 years of age) with dermatophyte infections of glabrous skin had 4-mm punch biopsies and skin surface biopsies (SSB) from an immediately adjacent site, at the periphery of the ringworm lesions. Eleven had *Trichophyton rubrum*, four had *Trichophyton mentagrophytes* and three *Epidermophyton floccosum*. Periodic acid Schiff (PAS)-stained histological sections were evaluated for the degree of eczema present, employing a visual analogue scale (VAS) scoring system. PAS-stained SSBs were examined for hyphal length as a function of density of infection using an eyepiece grid graticule and a stereological technique.

Mononuclear cells were found to predominate in the infiltrate in all biopsies but significant numbers of neutrophils and eosinophils were found in eight and seven biopsies respectively. There was a significant negative correlation between the duration of the infection and the mean hyphal density in SSBs ($r = -0{\cdot}48$, $P = 0{\cdot}001$) but no relationship was found between the degree of eczema in sections and the hyphal density in the stratum corneum. The different fungal species tended to be associated with different mean hyphal densities (*T. rubrum* 163·4 ± 149·6, *T. mentagrophytes* 114·0 ± 91·2, *E. floccosum* 102·7 ± 36·6) but there was no correlation of the degree of eczematization with fungal type. The data thus far suggest that host factors are more important in the expression of the characteristic eruption of ringworm rather than factors associated with the invading micro-organism

Epidermal eumelanin and phaeomelanin concentrations in different skin types and in response to PUVA.

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There are two types of melanin in mammals—the brownish-black eumelanin and the reddish-yellow phaeomelanin. Both pigments are present in human hair but there is no information on their relative amounts in human skin. In this study we have measured the concentrations of eumelanin and phaeomelanin in different skin types and in response to PUVA. Samples of epidermis were obtained from suction blisters raised in the upper arm of 12 Caucasian subjects (skin types I, II & III). The method of Ito and Fujita (*Anal Biochem*, 1986; **14**: 527–35) was used to measure eumelanin and phaeomelanin which were converted to pyrrole-2,3,5 tricarboxylic acid (PTCA) and aminohydrophenylalanine (AHP) respectively prior to analysis by HPLC. Eumelanin and phaeomelanin were found in all samples and the mean concentrations in skin types I ($n = 2$); II ($n = 5$) and III ($n = 5$) were 0·45; 1·24 ± 0·29 and 1·76 ± 0·46 ng PTCA/mg epidermis and 5·5; 3·78 ± 1·42 and 5·10 ± 0·89 ng AHP/mg epidermis respectively. The concentrations of both pigments increased following 6 weeks of PUVA therapy. The increased levels in the three skin types were 1·8, 1·98 ± 0·45 and 3·72 ± 1·19 ng PTCA/mg epidermis and 10·34, 5·24 ± 1·25 and 8·58 ± 1·00 ng AHP/mg epidermis. These results suggest that both eumelanin and phaeomelanin are present in the epidermis and increase in concentration following PUVA. The concentrations of these two pigments in the epidermis appear to be related to skin type and this is now being examined in a larger group of subjects.

Preliminary investigations into the production of mitogenic factors by cultured dermal papilla cells from human hair follicles. M.J.THORNTON,* K.ELLIOTT,† A.G.MESSENGER† AND V.A.RANDALL*, **Department of Biomedical Sciences, University of Bradford, Bradford, and* †*Department of Dermatology, Royal Hallamshire Hospital, Sheffield, U.K.*

The mesenchyme-derived dermal papilla of the hair follicle has a controlling influence on many parameters of hair growth. Since dermal fibroblasts produce diffusible substances that promote epidermal growth, we have investigated whether cultured dermal papilla cells from human hair follicles secrete mitogenic factors.

Conditioned medium (DP-CM) was prepared by incubating a confluent monolayer of dermal papilla cells in serum-free medium for 24 h. Cells were seeded into 24-well plates at 2×10^4 cells per well, and grown in media E199 with 20% fetal bovine serum for 3–4 days. After 48 h in 1 ml of serum-free media, cells (× 3) were treated with various media for 24 h, before incubation with $[^3H]$-thymidine (0·5 μCi well) in fresh media for 6 h. Cultures were washed with saline (× 2), treated with 10% trichoroacetic acid and solubilized with NaOH for radioactivity estimation.

DP-CM increased thymidine uptake per 10 cells by *c*.20 × in dermal papilla cells (23 media, five cell lines) compared to serum-free media, but by only *c*.5 × in dermal fibroblasts. Dermal papilla cells released *c*.1·5 × more soluble protein than dermal fibroblasts cultured from the same biopsies. When thymidine-uptake was related to the amount of protein, the effect of DP-CM was *c*.30 × higher in dermal papilla cells than normal growth media, but remained *c*.5 × in dermal fibroblasts. Uptake in DP-CM by beard dermal papilla cells was twice that of scalp cells (14 media, three cell lines).

These results demonstrate that dermal papilla cells secrete diffusible factors that stimulate cell proliferation. Dermal papilla cells are clearly more responsive to these factors than corresponding dermal fibroblasts; and the extent of their response may depend upon body site. Such factors may be important in regulating hair growth.

This research is supported by the Medical Research Council.

Sugars protect desmosomal glycoproteins from proteolysis. ANNETTE WALSH AND S.J.CHAPMAN, *Department of Dermatology, Royal Liverpool Hospital, U.K.*

Desmosomal adhesion proteins may be protected from proteolytic attack by their asparagine-linked, complex, oligosaccharide chains (Overton J., *Dev Biol* 1982; **92:** 66–72). To provide morphological evidence in support of this, pig-ear epidermis was exposed sequentially to hydrolytic enzymes, incubated individually for 48 h at 37°C, over 2 weeks. Experiments were repeated three times and specimens monitored by transmission electron microscopy.

The two major approaches were: (i) glycosidase treatment, to remove stepwise the sugar moieties in a typical complex oligosaccharide chain (neuraminidase, α-mannosidase, β-mannosidase, β-galactosidase, *N*-acetyl glucosaminidase); (ii) proteolysis using both endopeptidases (cathepsins B and D) and an exopeptidase (carboxypeptidase A). Each enzyme was used at its optimal pH and controls comprised specimens exposed to the appropriate sequence of buffers alone at 37°C.

Proteases alone induced no obvious changes in desmosomes or in the modified desmosomes (corneosomes) of the stratum corneum, when compared with controls. Glycosidases alone, or proteases followed by glycosidases caused a mild fragmentation of the intercellular structure of desmosomes. However dramatic changes occurred when glycosidase treatment was followed by proteolysis, characterized by extensive leaching and widening of desmosome and corneosome intercellular spaces. These findings indicate that sugar groups protect both desmosome and corneosome glycoproteins against proteolysis. However, whether removal of all sugars is required for proteolysis to occur remains to be determined.

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Pneumocystis carinii pneumonia following methotrexate

SIR, We were interested to read Drs Liddle and Marsden's letter (*Br J Dermatol* 1989; **120:** 582) concerning drug interactions with methotrexate, resulting in bone marrow failure. We have recently seen a patient who presented with *Pneumocystis carinii* pneumonia as a result of lymphopenia while on low-dose methotrexate.[1] She was also taking a non-steroidal anti-inflammatory drug (NSAID) and this was felt to be contributory to the methotrexate toxicity.

A 16-year-old girl, weight 38 kg, with long-standing and extensive cutaneous psoriasis complicated by arthropathy and unresponsive to several systemic therapies, was commenced on oral methotrexate 5 mg/week, gradually increasing to 10 mg/week over a period of 4 months. Subsequent control of her psoriasis was excellent and routine blood counts satisfactory. After a further 6 months the patient developed oral ulcers, epistaxes and a pancytopenia: leukocytes $1{\cdot}1 \times 10^9$/l (4–10), 70% neutrophils, 21% lymphocytes; platelets 48×10^9/l (150–400); haemoglobin 7·2 g/dl (12–16), RBC $2{\cdot}84 \times 10^{12}$/l (4–6·5) with normal indices. The serum IgG was reduced at 3·3 g/l (normal range 6–16 g/l) with other immunoglobulins and liver function tests normal and the serum creatinine was 50 μmol/l. Concurrent medication was ibuprofen 600 mg b.d. and prednisolone 1 mg/day, with the doses unchanged over the previous 12 months. Despite immediately stopping the methotrexate, the patient was admitted 1 week later with acute respiratory failure necessitating a tracheotomy and ventilation for a week. Bronchoalveolar lavage identified *Pneumocystis carinii* and the patient responded satisfactorily to high dose intravenous co-trimoxazole. The pancytopenia was recovering by the time of her admission with acute pneumonia. Subsequently the immunoglobulins, full blood count and peripheral lymphocyte subsets returned to normal despite containing the ibuprofen and prednisolone.

Pneumocystis carinii pneumonia as a consequence of low-dose methotrexate has been described in one other patient being treated for rheumatoid disease.[2] The low weight of the patient and the concomitant NSAID therapy, with its potential to reduce renal excretion, were considered to predispose her to methotrexate toxicity, that resulted in a near-fatal pneumonia.

We draw attention to this case to heighten awareness of the capacity of low-dose methotrexate to produce toxic effects on the bone marrow. We strongly reinforce the recent recommendations that full blood count should be monitored monthly in those receiving low-dose methotrexate,[3] especially when predisposed to toxicity by concurrent NSAID therapy. Patients should be warned of the symptoms of marrow failure when commenced on methotrexate, to minimize the risks of morbidity and possible mortality from this therapy.

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Book Review

Mycobacterial Skin Disease. Edited by M.HARAHAP (1989). Part of general series New Clinical Applications in Dermatology. Editor J.VERBOV. Dordrecht/Boston/London: Kluwer Academic Publications. Pp. 142. Illustrations 42 black and white. ISBN 0-7462-0119-2. Price £30.00.

The similarity between man and the nine-banded armadillo may not immediately spring to mind but *Homo sapiens* and his thick-skinned distant animal relation share the dubious distinction of being the only natural hosts of *Mycobacterium leprae*, the causative organism of leprosy. This fact along with full clinical and pathological accounts of all the mycobacterial skin diseases are contained within this useful publication.

The stated general aim of this series is 'to focus on developments in medicine which are now reaching the stage where they can be put into routine, daily clinical use.' The most recent advances in the ancient diseases of tuberculosis and leprosy have been in the development of practical, effective and shortened therapeutic regimens which have led to the taming of these once rampant conditions. These are clearly explained within the chapters dedicated to the clinical aspects of the two conditions, and include the recently introduced quadruple-agent therapy of tuberculosis and illustrations of specially designed calendar-blister packs for the administration of long-term anti-leprosy treatment which would be a boon to most clinicians trying to explain the complexities of these regimens. One significant omission however is the lack of information concerning the avoidance, detection and management of side-effects from anti-mycobacterial drugs, essential for clinicians dealing with patients on a day to day basis.

The bacteriology, immunology and clinical features are well documented and the photographs, although printed in black and white, convey the important physical signs. The photomicrographs are less clear however and this is disappointing in a book of this price. Useful chapters looking at the atypical mycobacterial skin diseases contain both good clinical and bacteriological advice.

The general arrangement of the chapters is logical but perhaps a little artificial, dividing the bacteriological and immunological aspects from the clinical features and necessitating a quick flick of pages if the reader wishes to digest a subject in its entirety. This is however a minor quibble and dermatologists will find this a useful publication when faced with these unusual cases. A personal copy would, I think be a little extravagant but I would recommend this publication as a useful addition to most medical libraries where its contents would be of interest to bacteriologists and infectious disease physicians as well as dermatologists.

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The discrimination between nickel-sensitive and non-nickel-sensitive subjects by an *in vitro* lymphocyte transformation test

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SUMMARY

A lymphocyte transformation test (LTT) was able to distinguish between nickel-sensitive subjects and non-nickel-sensitive controls. Sixty-one out of 66 (92%) nickel-sensitive subjects had positive stimulation indices in 6- and/or 7-day assays using 5 μg/ml of nickel sulphate, whereas none of the 43 controls gave positive results. Stimulation indices were not enhanced by the patch testing of subjects to nickel before performing the LTT. A weak correlation was seen between the results of the LTT and the macroscopic degree of patch-test reactivity. The concentration of nickel sulphate used (5 μg/ml) did not have a significant non-specific mitogenic effect.

Nickel is the commonest cause of allergic contact dermatitis in the UK.[1,2] Sensitivity to nickel is established at present by epicutaneous patch testing using 5% nickel sulphate in petrolatum. Patch testing rarely causes sensitization, but occasionally results in false positive and negative reactions, or the aggravation of existing dermatitis.[3] It is sometimes difficult to distinguish an irritant reaction from a true allergic response, and patients need to attend on three separate occasions.

Many authors[4–9] have reported the use of *in vitro* lymphocyte transformation tests (LTT) to discriminate between nickel-sensitive subjects and non-sensitive controls. Results have been variable and false positive reactions common. However, some of these false positive results[9] may have resulted from a non-specific mitogenic effect of nickel sulphate at higher concentrations.[7,8] Twenty-three out of 25 nickel-sensitive subjects were detected using an LTT, whereas none of the 10 controls were positive.[6] Al-Tawil *et al.*[8] described a set of criteria for detecting nickel sensitivity *in vitro* which required different nickel sulphate concentrations and direct

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comparisons with lymphocytes obtained from controls. They obtained positive results in 15 out of 16 nickel-sensitive subjects and in none of nine controls.

We have used 6- and 7-day LTTs to compare the response of lymphocytes taken from nickel patch test-positive subjects and from non-nickel-sensitive controls.

METHODS

Subjects

Sixty-six subjects (58 female, eight male; mean age 33 years) had positive allergic patch-test reactions to 5% nickel sulphate ($NiSO_4$ $7H_2O$) in petrolatum. Patch tests were read at 48 and 120 h. Reactions were scored for their severity on the scale according to Wilkinson *et al.*[10] (− no reaction, +? doubtful, + weak, + + strong, + + + extreme).

Controls

Forty-three controls (32 female, 11 male; mean age 35·7 years) were studied. Thirty-seven were healthy volunteers who had no history of metal contact reactions; six controls (C1–6) were subjects attending the patch test clinic with histories suggesting nickel sensitivity, but who were negative on patch testing.

Tissue culture medium (TCM)

To RPMI 1640 medium was added 100 IU/ml penicillin, 100 μg/μl streptomycin, 2 mmol/l L-glutamine, 0·08% sodium bicarbonate (Gibco, Paisley), and 2×10^{-5} mol/l 2-mercaptoethanol (Sigma Chemicals, Dorset).

Stimulants

Concanavalin A (Con A), (Sigma Chemicals) was used at a final concentration of 10 μg/ml. A 1% nickel sulphate ($NiSO_4$ $7H_2O$) (Analar, BDH Chemicals, Glasgow) solution was prepared in physiological saline, sterilized and stored at 4°C. The final concentrations ranged between 0 and 50 μg/ml of nickel sulphate.

Isolation of lymphocytes

Blood (20 ml), obtained by venepuncture and mixed with heparin (10 IU/ml), was layered onto lymphocyte separation medium (Flow Laboratories, Herts) and centrifuged (400 ***g***, 20 min, room temperature). The separated plasma was added to TCM to give a final concentration of 15% (TCM-P). The mononuclear cells (PBM) were washed in PBS, then resuspended at a concentration of 10^6/ml in TCM-P.

The lymphocyte transformation test (LTT)

Cells were stimulated by the addition of either 10 μg/ml of Con A or 0–20 μg/ml of $NiSO_4$. Cells (2×10^5) were incubated in a round-bottomed Linbro tissue culture plate (Flow Laboratories, Herts) in 200 μl of TCM-P. Cells were cultured at 37°C in an atmosphere containing 5% CO_2 for a total of 6 or 7 days.

Lymphocyte transformation was measured by the incorporation of ^{3}H-thymidine. Radiolabel (1 μCi) was added to the cells for the final 18 hours of culture. Cells were harvested onto Skatron filter discs using a MASH II harvester and the incorporated radioactivity was measured using a liquid scintillation counter. Proliferation was expressed as the mean counts per min (cpm) ± standard deviation for quadruplicate cultures, and stimulation indices (SI) were calculated (cells + stimulant (cpm)/cells alone (cpm)). SI $\geqslant 3{\cdot}0$ were considered to indicate nickel sensitivity.

RESULTS

Detection of nickel sensitivity using the LTT

The 6-day LTT was used to obtain dose–response curves using 0–20 μg/ml of nickel sulphate for 66 nickel-sensitive subjects and 43 controls. Nickel sulphate concentrations greater than 30 μg/ml had previously been shown to be toxic to the cells (data not shown). Fifty-six of the nickel-sensitive subjects and all controls were also studied in the 7-day LTT.

Representative results from four nickel-sensitive subjects and four controls in a 6-day assay are shown in Figure 1. One control showed a large response against 20 μg/ml of $NiSO_4$, indicating a mitogenic effect by the $NiSO_4$ at this concentration. Similar results were obtained in the 7-day assay. Using the criterion of SI $\geqslant 3$ indicating a positive result, 13 of the controls were positive using 20 μg/ml $NiSO_4$ and five controls were positive when 10 μg/ml $NiSO_4$ was used (data not shown).

As expected, there were no statistical differences in the proliferation of unstimulated cells or of cells stimulated with the mitogen Con A, when comparing the uptake of ^{3}H-thymidine into cells taken from nickel-sensitive subjects and from controls (data not shown).

Figure 2 shows the SI (median + interquartile range) obtained using 5 μg/ml of $NiSO_4$ for the 43 controls and all of the nickel-sensitive subjects whose patch-test results were known. Excluded from this figure are the SI of three patch-test positive patients where the patch-test score was unknown. The SI were $\geqslant 3{\cdot}0$ for 56/66 nickel-sensitive subjects in the 6-day assay. Six sensitive subjects were positive only in the 7-day assay; and two subjects only in the 6-day assay. Together, the 6- and 7-day assay gave positive results for 61 out of 66 (92%) of the nickel-sensitive subjects. Using the criterion of SI $\geqslant 3{\cdot}0$, none of the controls were positive in either the 6- or 7-day assay.

Five nickel-sensitive subjects were not detected by the two assays. Four of these subjects reported reactions to cheap jewellery. Three of these individuals had weak positive (+) patch-test reactions to nickel sulphate. Only one subject had an extreme (+ + +) patch-test reaction.

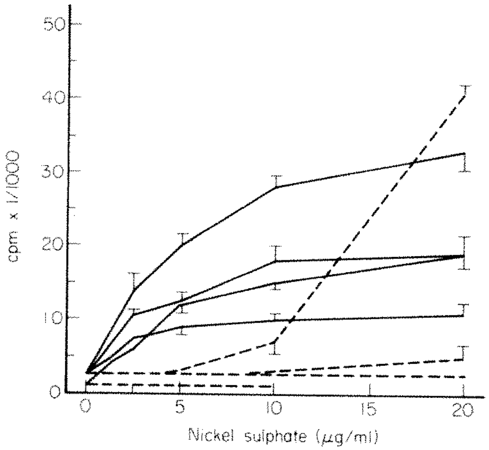

FIGURE 1. Nickel sulphate dose–response curves in 6-day assay: PBM from nickel-sensitive patients (——); PBM from non-sensitized controls (– – –).

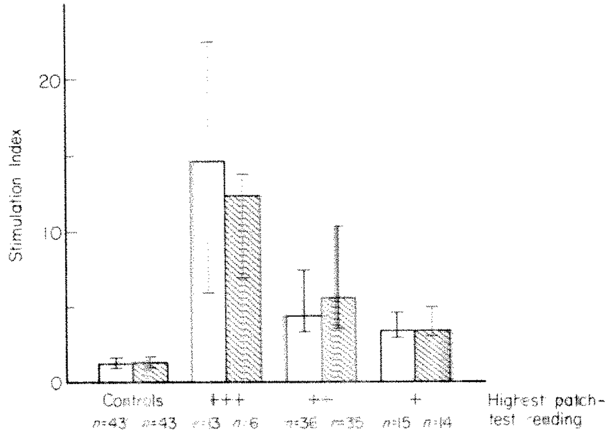

FIGURE 2. Patient stimulation indices obtained with 5 μg/ml $NiSO_4$ and patch-test results (median + interquartile range): 6-day LTT (☐); 7-day LTT (▨). Patch test positive but score unknown, $n = 3$.

Failure to detect this subject in the 7-day assay may have resulted from an abnormally high background incorporation of ^3H-thymidine (14 000 cpm). Significant differences between cells from nickel-sensitive subjects and controls were obtained for nickel sulphate concentrations between 2·5 and 20 μg/ml ($P < 0{\cdot}001$; Student's *t*-test) in both the 6- and 7-day assays.

LTT results are not enhanced by patch testing

Blood samples were taken from most subjects when they attended the patch-test clinic for their second reading (at 120 hours). To ensure that the patch testing itself did not enhance the LTT, blood was obtained from six subjects immediately before patch-test application and again when they attended the clinic for their second reading.

The SI obtained are shown in Figure 3. Results obtained pre- and post-patch testing were not significantly different using Wilcoxon's Rank Sum Test.

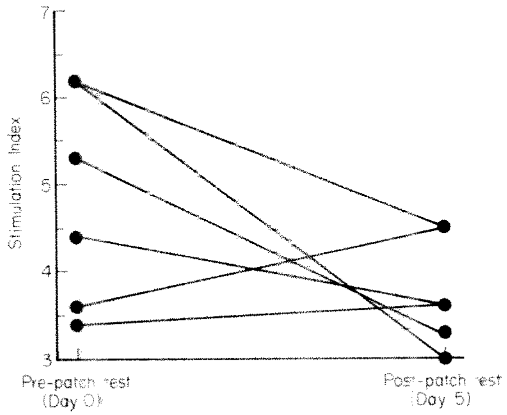

FIGURE 3. Stimulation indices before and after patch testing.

Correlation between patch test and LTT results

Having established that the LTT is capable of detecting the majority of nickel-sensitive subjects, the Wilcoxon's Rank Sum test was used to evaluate the correlation between patch-test scores and the SI obtained using 5 μg/ml $NiSO_4$. Correlation coefficients of 0·38 and 0·33 were obtained for patch-test scores with 6 and 7-day LTT results, respectively, indicating that the correlation between the grade of patch-test positivity and size of SI was weak.

DISCUSSION

The LTT with nickel sulphate was first described in 1970.[4] Since then, the ability of the LTT to detect nickel sensitivity has been variable. Two studies[6,8] described assay methods detecting at least 92% patch-test positive subjects with no false positive results. However, using a simple method which would be suitable for routine use, we have studied a larger number of patients and controls. Five nickel-sensitive subjects were not detected in our assay; three of these had had very weak positive patch-test reactions which may have been irritant in nature. It is also possible that the cells obtained from these individuals may have contained insufficient numbers of circulating dendritic cells. The addition of either enriched blood dendritic cells or Langerhans cells to the patients' T cells may have stimulated nickel-specific proliferation.[11]

No false positive results were obtained using the assay. Technical differences, for example the density media used to isolate the PBM[12], may contribute to the differences between our results and those reported previously. Furthermore, concentrations of $NiSO_4$ greater than 5 μg/ml were found to stimulate cells from certain non-sensitized individuals. These higher concentrations may have contributed to the false positive results in a study using 32·3 μM (approximately 10 μg/ml) in most of the assays.[9] A further 'blind' prospective study using cells from similar numbers of patients and controls before patch testing will indicate whether the assay can replace patch testing effectively.

It is unlikely that patch testing enhanced the LTT results. In nickel-sensitive subjects, similar results were obtained in LTTs performed either 5 days after patch-test application or immediately before patch testing. This contrasts with the findings of Al-Tawil *et al.*[13] who showed that the SI from the cells of two sensitive subjects increased 2 and 7 days after patch testing and then began to decrease (14 days).

The correlation between patch-test results and SI in the LTT was weak with correlation coefficients of 0·38 and 0·33 for the 6- and 7-day assays. A weak correlation was also shown by Al-Tawil *et al.*,[14] in studies comparing LTT results with a serial dilution patch test. They suggested that patch-test reactions could result from mechanisms other than those mediated by antigen-specific T cells, such as the release by mast cells of vasoactive amines. Others have suggested that negative *in vitro* reactions may occur if nickel-specific cells are retained at a particular anatomical site, but in our view this is unlikely.

False positive patch-test reactions of an irritant nature may occur with several potential allergens including nickel, and are sometimes observed in atopic subjects.[3] It is not always easy to distinguish an irritant from a true allergic reaction from the appearance of a patch test. Apart from the complication of false positive or negative reactions, patch testing may induce adverse reactions that include active sensitization, 'the angry back syndrome', alterations in skin pigmentation and reactivation of dermatitis. The use of an *in vitro* method to detect nickel sensitivity overcomes these difficulties. Furthermore, a patient needs to attend the hospital only once instead of three times. We have shown that the employment of a 6- and 7-day LTT using 5 μg/ml $NiSO_4$ (with an SI $\geqslant 3{\cdot}0$) will establish nickel sensitivity as efficiently as patch testing in at least 90% of cases without the complication of false negative or false positive results due to toxic or mitogenic effects. The development of similar assays for other allergens could eventually replace patch testing, at least for the most common substances.

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Nickel-sensitive patients with vesicular hand eczema: oral challenge with a diet naturally high in nickel

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SUMMARY

Oral challenge with nickel sulphate indicated that vesicular hand eczema in nickel-sensitive patients may be exacerbated by nickel occurring naturally in the diet. Twelve nickel-sensitive females with vesicular hand eczema were challenged with a supplementary high nickel diet for 4 days in a single-blind cross-over study. The diet had about five times the average nickel content of the daily Danish diet. An aggravation of the hand eczema was observed in six out of 12 patients on day 4 after the start of the challenge. By day 11, the hand eczema was worse in 10 out of 12 patients, and remained unchanged in two.

The prevalence of nickel sensitivity is about 8–14·5% for adult women and about 1% for men as evaluated by patch testing or history in the United States, Finland and Denmark.[1–5] The incidence of nickel sensitivity has doubled in all age groups from 1948 to 1973[5] and about half the women have hand eczema.[1,3,5–6]

An oral dose of 5·6 mg of nickel in a double-blind study produced an exacerbation in nine out of 12 subjects with hand eczema of the pompholyx type, with none reacting to the placebo.[7] In contrast, handling of nickel-containing metal objects did not induce eczema, indicating that the ingestion of nickel was of greater importance.[7] This was confirmed in subsequent studies using doses of 2·5–5·6 mg,[8–11] though other studies showed no exacerbation following oral provocation using 2·5 mg or lower doses.[12–14] The latter studies were double-blind with weeks of nickel supplementation and weeks on placebo, randomly following each other. However, evaluation of the eczema status was performed within less than 3 days after exposure. The dose of nickel given in these studies was at or above the normal daily intake of 100–300 μg/day[15–17] and up to 900 μg/day in extreme situations.[18] A diet high in nickel often includes cocoa or

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chocolate, soya beans or other dried legumes, different types of nuts (walnuts, almonds, hazel nuts), and oat meal. These foodstuffs all have a nickel content higher than about 1 μg/g wet weight.[17-18]

In this study we investigated whether oral intake of nickel naturally occurring in the diet can aggravate vesicular hand eczema in nickel-allergic patients.

METHODS

Patients

Fifteen nickel-allergic patients were selected using the following inclusion criteria: a history of dermatitis from skin contact with 'white metal' objects; a positive patch test to 5% nickel sulphate in petrolatum within the last 2 years; present vesicular hand eczema of the pompholyx type; and, no history or clinical signs of atopic dermatitis. Patients with irritant contact dermatitis were excluded. All the patients were patch tested with the International Contact Dermatitis Research Group (ICDRG) standard series of contact allergens (TROLAB) including nickel sulphate 5%. Where patch tests had been carried out more than 2 years previously, the patients were tested for confirmation after termination of the study. Finn Chambers (Epitest Ltd, Helsinki, Finland) on Scanpor (Norgesplaster A/S, Oslo, Norway) were used. The patches were applied for 48 h and read at 72 h according to the ICDRG recommendations.[19] Reactions + to + + + were recorded in all patients. Three of the patients had positive reactions to cobalt, one to formalin and one to neomycin. Three patients were excluded during the study: two because of disease not related to the study and one because of failure in follow-up. The age range of the 12 patients who completed the study was 21–64 years, median 35 years. The duration of their vesicular hand eczema was 1–13 years, with a median of 5 years. In all, the eczema was located on the fingers and in 10 the palms were also involved. During the study, the patients were allowed to use topical steroid preparations and an emollient. Three patients (Nos 1, 3, 8) had some years ago been treated with disulfiram because of extensive eczema.[20] They had responded well to this treatment, but the drug was withdrawn because of hepatotoxicity in two patients.

Supplementary diet

The supplementary diet was prepared from the following food items (the nickel content was calculated from published values[17] and is given in square brackets): 100 g oatmeal [176 μg Ni/day]; soya bean stew (100 g soya beans, 90 g carrots and 80 g leeks) [532·4 μg Ni/day]; chocolate cake (39 g wheat flour, 39 g margarine, 39 g sugar, 39 g egg, 13 g milk, 13 g cocoa, 1·3 g baking powder) [150·6 μg Ni/day]. The diet contained 6151 kJ/day (1469 kcal/day), and the calculated energy distribution[21-22] was as follows: fat 40%, carbohydrates 43%, protein 18%. All foods were bought simultaneously in the same store with the same production date for each type of food. The oat meal and soya beans were thoroughly mixed before use to ensure the highest degree of homogeneity. Samples of each type of food were analysed for nickel content by the National Food Agency, Søborg, Denmark. The samples were pretreated in a Berghuf pressure digestion system. The nickel content in the sample was determined by electrothermal atomic absorption spectroscopy (Perkin-Elmer 3030 with Zeeman background correction). Contents were estimated by the standard addition method (adding 50% and 100% of expected contents). The detection limit, estimated as three times the variation of the blank, was 0·10 μg/g. For every five samples, blanks and standard reference material were determined. The nickel concentration in NBS 1571 Orchard Leaves was found to be 1·17 ± 0·08 μg/g (certified value 1·3 ± 0·2 μg/g).

Urine samples

Twelve-hour urine samples were collected directly in acid-washed wide-mouth polyethylene bottles with screw-caps (2000 ml). The samples were pretreated and analysed according to the method described by Sunderman *et al.*[23] The equipment used was a Perkin-Elmer model 5100 atomic absorption spectrometer with a Zeeman background correction, an HGA-600 graphite furnace and an AS-60 autosampler. The concentration of nickel in the urine was determined by using the method of standard addition. The samples were mixed in a 1 : 1 ratio with standards consisting of Triton X-100 (0·5 g/l), nitric acid (0·1 mol/l) and nickel chloride (0, 5, 10 and 15 µg/l, respectively) in water from a Millipore Q system. The detection limit estimated as three times the variation of the blank was 0·55 µg/l. The total analytical imprecision in nine determinations was estimated to be 12·6%, 9·0% and 4·6% at urine levels of 2·5, 3·5 and 8·5 µg/l, respectively. The accuracy of the nickel determinations was ensured using Lanonorm® Metalle 1 (Behringwerke AG, Marburg, W.Germany) as a quality control. The average nickel concentration in Lanonorm® Metalle 1 was found to be 3·53 ± 0·11 µg/l (assigned nickel value, 3·4 ± 0·6 µg/l). The urine creatinine was determined by the conventional Jaffé-reaction on an Auto Analyzer II (Technicon).

Design

The study design is shown in Figure 1. All patients went through a preliminary examination 14 days before the start of the study. The patients were randomized using random numbers,[24] where even and uneven numbers referred to the two groups. After the initial observation period of at least 14 days, one group was given the supplementary diet for 5 days of the first week, after which they resumed their normal dietary habits during the second week. The other group continued their normal diet during the first week and were given the supplementary diet for 5 days of the second week. The patients were examined on the first and fifth days of both weeks and on the fifth day of the third week. This ensured the examination of all the patients on days 14 or 21 before start of provocation with the supplementary diet and on days 0, 4 and 11 after start of the provocation period, while challenge lasted from day 0 to day 4. Four patients (Nos 2, 3, 7 and 11) were not able to eat the total amount of the supplementary diet. Urine samples were collected over a 12-hour period from all the patients during both weeks of challenge. Accordingly, urine collection proceeded as follows. Group 1: nights before days 0, 4, 7 and 11. Group 2: nights before days −7, −3, 0 and 4. The activity of the hand eczema was assessed as follows: (a) The patient's subjective evaluation using a linear scale from no eczema (0 mm) to severe eczema (120 mm). (b) The number of vesicles in a defined area of the palm of the hand with the highest number of vesicles at the first examination (Fig. 2). When vesicles were present on the fingers, the one with the highest number was also counted. (c) Clinical description of the

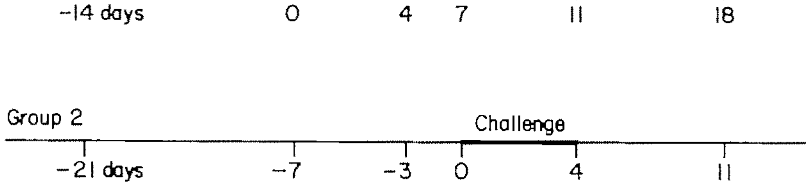

FIGURE 1. Study design for the single blind cross-over study with 12 patients randomly divided into two groups Examinations of all patients were performed 2–3 weeks before challenge, and on days 0, 4 and 11.

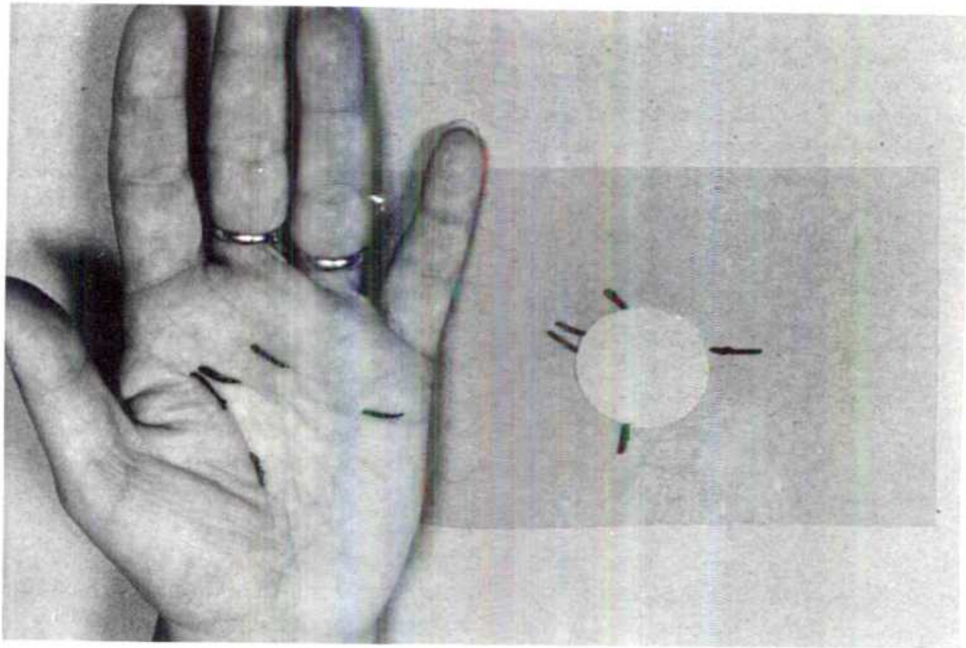

FIGURE 2. Template with markings referring to the lines of the hand.

eczema. From these descriptions, the eczema was retrospectively classified into eight classes of increasing severity. All assessments were performed by the same dermatologist (LVJ) who was unaware of the patient's diet.

The studies were carried out in accordance with the Helsinki convention and notified to the Scientific Ethical Committee for Vejle and Funen counties.

RESULTS

The nickel content of the supplementary diet, both the amount calculated from literature sources and the much lower content indicated by the chemical analysis are shown in Table 1.

Six patients were challenged during the first week of the study and the other six during the second week. The urinary nickel excretion increased in all patients, including four patients who were unable to eat the whole diet. This increase averaged about fourfold from day 0 (median, 0·73 μg Ni/g creatinine; range <0·43–3·15 μg Ni/g creatinine) to day 4 (median, 2·78 μg Ni/g creatinine; range 1·19–6·33 μg Ni/g creatinine) ($P = 0{\cdot}0002$, Wilcoxon test). Table 2 shows the

TABLE 1. Nickel content (μg/day) of supplementary diet

	Ni	Analysed	Calculated
Oatmeal (100 g)	10	53·9 ± 4·6	176
Soya bean stew	9	324·6 ± 70·1	532·4
Chocolate cake	10	113·1 ± 20·9	150·6
Total		491·6	859

TABLE 2. Urinary nickel excretion in two groups of six patients during the study period (total Ni in μg/12 h and adjusted to the creatinine excretion). Supplementary intake of a diet high in nickel was given on days 0–4

Days			−7	−3	0	4	7	11
μg Ni/12 hours	Group 1	Median			<0·55	1·62*	0·88	<0·55
		Range			<0·55–2·24	0·69–2·40	<0·55–1·70	<0·55–1·20
	Group 2	Median	<0·55	<0·55	<0·55	1·86*		
		Range	<0·55–0·73	<0·55–1·48	<0·55–0·58	0·81–2·52		
μg Ni/g creat†	Group 1	Median			0·83	2·53*	1·83	0·78
		Range			0·45–3·15	1·19–4·71	0·50–2·32	0·45–2·26
	Group 2	Median	<0·95	<0·70	<0·71	3·05*		
		Range	<0·49–1·22	<0·36–1·8	<0·43–1·07	1·29–6·33		

*$P = 0{\cdot}0002$ for a significant increase from day 0 to day 4 (Wilcoxon test).

†The results may be converted to μmol Ni/mol creatinine by multiplying by 1·93.

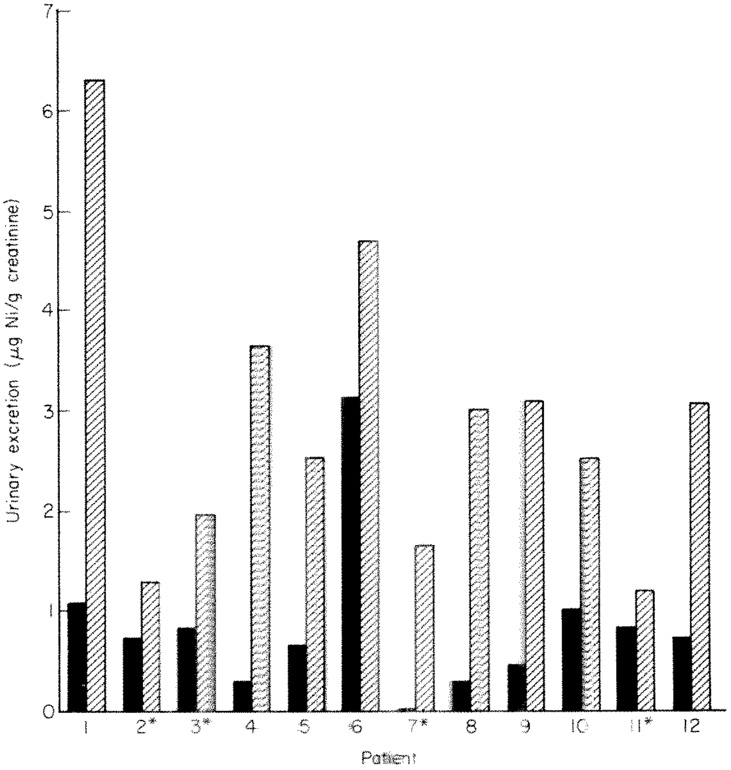

FIGURE 3. Urinary nickel excretion in each of the 12 patients on days 0 (■) and 4 (▨). * Unable to eat the whole diet.

TABLE 3. The patients' own evaluation of eczema status on a linear scale

	14 or 21 days before start	Day 0	Day 4	Day 11*
1	4	12	43	56
2†	79	24	82	39
3†	49	63	5	65
4	61	59	61	60
5	44	38	31	59
6	61	61	90	86
7†	56	30	24	68
8	48	82	108	113
9	30	5	37	82
10	21	9	14	13
11†	32	72	80	86
12	34	25	23	47

* $P = 0{\cdot}01$ (Friedman test).

† Unable to eat the whole diet.

urinary nickel excretion in μg Ni and μg Ni/g creatinine. The results can be converted from μg Ni/g creatinine to μmol Ni/mol creatinine by multiplying by 1·93. Figure 3 shows the urinary nickel excretion on days 0 and 4 for each of the 12 patients.

Table 3 shows the patients' evaluation of eczema status. On day 4, eight patients indicated aggravation and four improvement. All patients were worse by day 11. No significant difference in the degree of eczema was seen from day −14/21 to day 0 and from day 0 to day 4. However, the worsening of the eczema from day 0 to day 11 was highly significant ($P=0{\cdot}01$, Friedman test).

The number of vesicles counted are shown in Table 4. In one case, the hand with the highest number of vesicles changed from the left to right after which both were counted. In some cases it was impossible to count the number of vesicles and these are indicated in the Table. In six of the patients, the number of vesicles increased by day 4, in two they decreased and in four the number was unchanged. On day 11, the vesicle counts were greater in 10 patients and lower in

TABLE 4. The number of vesicles in a defined area of the palm of the hand with the highest activity in eczema

		14 or 21 days before start	Day 0	Day 4	Day 11*
1		35	85	80	45
	f:	+	+	+	+
2†		0	0	15	15
	f:	0	0	0	0
3†		0	0	0	0
	f:	30	0	0	10
4		30	35	40	50
	f:	15	3	15	15
5		25	40	25	20
	f:	+	0	4	+ (10)
6		20	0	2	15
	f:	25	0	45	0
7†		15	0	0	0
	f.	0	0	0	8
8		30	35	40	45
	f:	50	50	many	many
9	L:	65	20	25	75
	R:			45	80
	f:	20	0	3	5
10		40	0	0	20
	f:	11	0	0	0
11†		20	50	80	85
	f:	20	80	100	100
12		0	0	0	0
	f:	0	0	0	many

L, left palm; R, right palm; f, finger.
many, impossible to count as too close together.
+, impossible to count due to weeping eczema and scratching.
* $P=0{\cdot}03$ (Friedman test).
† Unable to eat the whole diet.

TABLE 5. Dermatologist's evaluation of the overall eczema status (related to day 0) in 12 patients who ingested a nickel-rich diet on days 0–4

	Better	Worse	Same
Day 14/21 before	1	7	4
Day 4	0	7	5
Day 11*	0	12	0

* $P = 0{\cdot}001$ (Friedman test).

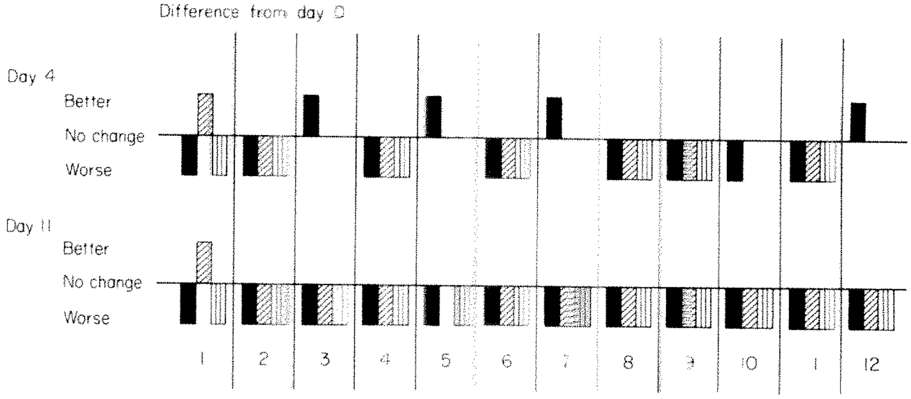

FIGURE 4. Concensus evaluation of eczema status. ■ = patient; ▨ = vesicles; ▥ = dermatologist.

two patients. However in the two latter patients, the eczema may have worsened; in the case of patient 5 the vesicles could not be counted due to scratching, and in patient 1 the skin of the hands was atrophied after many years of treatment with local steroids, and deep fissures were present. No change was seen from day 14/21 before start of challenge to day 0 and from day 0 to day 4, but there was a significant worsening from day 0 to day 11 ($P = 0{\cdot}03$, Friedman test).

Table 5 shows the evaluation by the dermatologist on a scale from 0 to 7, with 0 indicating no eczema and 7 indicating severe eczema. No change was observed from day 14/21 before start of challenge to day 0 and from day 0 to day 4, whereas a worsening was observed at day 11 ($P = 0{\cdot}001$ Friedman test). The evaluation of the eczema for each of the 12 patients is shown in Figure 4. On day 4 after challenge, the eczema in six patients was considered to have worsened, and by day 11, 10 of the 12 patients were definitely worse. The remaining two were subjectively worse and were evaluated as such by the dermatologist, but the number of vesicles was lower.

DISCUSSION

The discrepancy between the calculated and analysed nickel content of the dietary supplement is most likely due to the recent improved technology using atomic absorption spectrometry and to less contamination in the laboratory. The observed fourfold increase in nickel excretion after provocation corresponds well to the estimated four- to fivefold increase in dietary nickel intake. Assuming that this increase is about 400 μg Ni/day, that physiological variations in nickel excretion are minimal, and that all absorbed nickel is excreted in the urine within 1–2 days, then the intestinal nickel absorption is calculated to be about 1%. This compares well with the findings in other studies of nickel absorption when it is added to food.[25]

Two major conclusions can be drawn from the results shown in Tables 3–5. Firstly, a diet naturally high in nickel can result in a worsening of hand eczema in nickel-allergic patients. This observation is in agreement with most results obtained using oral doses of 2·5–5·6 mg inorganic nickel.[8-12] However, the doses of nickel, given as sulphate, were several-fold higher than those used in this study. As they were given between meals, it is probable that they were absorbed better.[25] The particular form of nickel in food, or the intake of the supplementary diet every day for four days, may be of importance for the aggravation of the eczema as seen in this study. Although other compounds contained in the supplementary diet might have caused the exacerbation, it is probable that the nickel was responsible. The second finding of importance is that the hand eczema was worse by day 11 and that no significant change was seen on day 4. This late reaction may explain why some double-blind studies[12-14] did not find any change during 3 days of observation.

Our results support the suggestion that a diet low in nickel is beneficial in the treatment of patients with vesicular hand dermatitis and nickel allergy.[8,26,27] However, the low nickel diet used in previous studies included almonds, oatmeal and dried legumes which have been shown to have a high nickel content.[18,28]

The response to oral intake of inorganic nickel may not be uniform and hyposensitization may occur. In two controlled studies,[29,30] patients with contact allergy to nickel were given oral nickel to hyposensitize them. Inorganic nickel in doses of 5·0 mg taken once a week for 6 weeks significantly lowered the degree of contact allergy, as measured by patch-test reactions before and after nickel administration, but 0·5 mg nickel daily did not have any influence on the degree of contact allergy.[29] Adaptation to oral doses of 2·24 mg inorganic nickel occurred after the patients were given gradually increasing daily doses over 3 months.[30] From our present study, we recommend that nickel-allergic patients with severe vesicular hand eczema should have their nickel intake lowered and the patients should be given appropriate dietary advice.

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HLA-A, B, C and DR antigens in nickel contact sensitivity

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SUMMARY

The relationship between HLA antigens (A, B, C and DR) and nickel contact sensitivity was examined in 54 patients with contact allergy, as confirmed by an unequivocal positive patch-test reaction only to nickel. A control group was 320 healthy blood donors from the same geographical area as the patients. The HLA-A, B, C and DR antigens were typed using standard serological methods. HLA typing revealed a significant increase of HLA-DRw6 antigen in the patient group (corrected $P < 0{\cdot}025$) and the relative risk for patients with DRw6 to develop nickel sensitivity was 3·32.

Nickel contact sensitivity is a classical form of delayed hypersensitivity, particularly in women and usually produces a chronic eczematous eruption at the sites of metal contact and/or on the hands.

Allergic contact dermatitis is an inflammatory reaction mediated by type IV hypersensitivity. In guinea-pigs and mice, the genes that control the delayed hypersensitivity reactions have been shown to be in linkage with those of the major histocompatibility antigens.[1–3] The immune response genes (Ia) of mice have their homologues in the human HLA-DR locus. The DR antigens are found only on cells capable of eliciting the mixed lymphocyte culture reaction[4] and, in the normal epidermis, only the Langerhans cells have HLA-DR antigens on their surface.[5,6] The HLA-DR antigens of Langerhans cells play a role in their antigen-presenting capacity[7] and, since these cells are involved in cell-mediated immune reactions, it may be possible to show linkage between the HLA-DR system and allergic contact dermatitis.

Several attempts have been made to establish linkage between HLA typing and nickel contact sensitivity in human beings, with conflicting results. In a preliminary study in which HLA was typed for A and B locus antigens, in a series of Swedish patients[8] it was suggested there was an association of HLA-B7 with nickel allergy, but the result was not corrected for the number of

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antigens. Further studies in Sweden[9] and in a twin-and-patient population group in Denmark[10] failed to demonstrate such an association between nickel allergy and HLA antigens of the A and B loci. In contrast, a British study of HLA-A and B antigens,[11] found an increased antigen frequency of HLA-B21 in a population of 39 nickel-sensitive females, but the relative risk (RR) for their patients was not estimated. A Norwegian study[12] showed no association between HLA-A, B, C and DR antigens and nickel sensitivity. A similar lack of association was found in a Finnish study[13] between HLA-A, B, C and D antigens and a population of atopic and non-atopic nickel contact dermatitis patients. A British study[14] in which HLA was typed for A, B and DR locus antigens, showed that B35 and Bw22 antigen frequencies and RR values were increased in 26 nickel-sensitive females in the Yorkshire region. In the same patient population the frequency of DR2 and DRw6 were increased but not to a significant degree. A study of the frequencies of HLA-A, B and DR antigens in 67 British patients with multiple-contact allergies[15] failed to show any significant HLA associations, although there was an increased frequency of DR4 in a subset of 36 patients who included nickel as one of their sensitivities, and an increased frequency of DRw6 in a subgroup of 20 patients sensitive to nickel alone.

To our knowledge, this is the first Italian study which has investigated a possible relationship of the HLA-A, B, C and DR locus antigens with nickel contact sensitivity.

METHODS

Patients

Fifty-four patients with nickel contact dermatitis were selected from those seen from November 1987 to November 1988. The clinical diagnosis of contact allergy was confirmed by an unequivocal positive patch test only to nickel when the patients were tested with a standard series of 27 allergens. The epicutaneous tests were carried out using Finn Chambers which were applied to the patient's back for 48 h. Only unequivocal positive tests were accepted and all were judged to be clinically relevant. The tests were read 48-96 h after application. The clinical aspects of contact dermatitis in our patients were eczematous reactions at sites of metal contact in 34 patients, and the other 20 patients had hand eczema. Of our 54 patients, 50 were female and four male, their ages varied from 14 to 55 years (mean 26 years) and all lived in the same area in northern Italy. The control group consisted of 320 healthy unrelated blood donors, of both sexes who were not patch tested, but had no past or present personal history of atopy or contact dermatitis and were from the same geographical area. Patients with positive patch-test reactions to other allergens in addition to nickel were excluded, as were patients with diabetes mellitus, psoriasis, lichen planus,[16] dermatitis herpetiformis and patients with past or present atopy (atopic dermatitis, allergic rhinitis and allergic asthma).

HLA typing

HLA typing was performed using the conventional NIH two-step complement-dependent microlymphocytotoxicity test on peritumoral lymphocytes or B-enriched lymphocyte preparations using AET-rosetting, with 150 antisera able to resolve 13 HLA-A, 25 HLA-B, 6 HLA-Cw and 7 HLA-DR antigens. All the typing was done by the same laboratory.

Analysis of data

The data were analysed by the chi-squared test with Yates' correction, to compare antigen frequencies in patients and control subjects. Probability (*P*) values were then corrected for the number of specificities tested.[17] The value of the strength and rank of association were obtained by calculating the relative risk (RR), a measure of the increased risk of developing the disease conferred by the genetic marker, using Woolf's method.[18]

RESULTS

The HLA-A, B, C and DR types are summarized in Table 1, with the calculated chi-squared, *P*, corrected *P* values and RR.

There was no difference between patients and controls for the alleles at the A and C locus, while for the alleles at the locus B we found non-significant differences in B5 (12·96% in patients vs. 21·25% in controls), B13 (15·56% in patients vs. 7·24% in controls) and in B14 (12·96% in patients vs. 6·25% in controls).

However, for the DR antigens we found a significant increase in DRw6 (37·7% in patients vs. 15·6% in controls), with $P < 0{\cdot}0005$ and corrected $P < 0{\cdot}025$. The RR for patients with DRw6 of developing nickel contact sensitivity was 3·32.

TABLE 1. Results of serological HLA-B and DR typing in nickel-sensitive patients and controls. The results of HLA-A and C typing have been omitted since there is no difference between patients and controls

HLA antigens	Controls ($n = 320$) (%)	Patients ($n = 54$) (%)
B5	21·25	12·96
B7	11·83	14·81
B8	11·64	11·11
B12	18·44	11·11
B13	7·24	15·56
B14	6·25	12·96
B15	7·84	9·26
B16	11·26	9·26
B17	8·60	9·26
B18	17·02	16·67
B21	9·56	7·41
B22	4·95	5·56
B27	4·17	3·70
B35	29·70	33·33
B40	5·91	3·70
DR1	16·31	16·98
DR2	22·22	28·30
DR3	18·02	16·98
DR4	12·17	13·21
DR5	35·95	33·96
DRw6	15·63	37·74*
DR7	24·19	24·53

* Chi-squared = 13·17, $P < 0{\cdot}0005$, corrected $P < 0{\cdot}025$, RR = 3·32.

DISCUSSION

The aim of this study was to determine the relationship between HLA antigens and nickel contact sensitivity, and the possible risk for the development of nickel allergy. In order to minimize complicating factors, we used a selected population composed of non-atopic patients with a contact sensitivity only to nickel and without an occupational exposure and those diseases that may be associated with certain HLA antigens. Since the patch test was not performed on the controls, it is possible that 5% of the control group were sensitive to nickel, as in the general population. However, since none of them was atopic this small number should not have influenced the results of this study.

Our study of HLA typing shows a statistically significant increase in the frequency and RR values of DRw6 antigen in nickel-sensitive patients. Our data are in partial agreement with previous studies,[14,15] but we found no significant association between HLA-A, B and C antigens and nickel contact sensitivity.

There is evidence to suggest that HLA-DRw6 is a marker for enhanced immunoresponsiveness for the production of endothelial/monocyte antibodies,[19] the rejection of a kidney graft and the formation of anti-class II antibodies after renal transplantation,[20] and the production of antibodies against streptococcal antigens.[21] Our results suggest that, in an Italian population, the DRw6 antigen could be a genetic marker for the likelihood to develop sensitivity to nickel, and possibly other sensitivities that may be detected in the future.

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Immunohistochemical identification of interleukin 1α and β in human eccrine sweat-gland apparatus

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SUMMARY

Bouin-fixed paraffin sections or acetone-fixed cryostat sections were labelled with the avidin–biotin complex (ABC) or peroxidase–antiperoxidase (PAP) method using three monoclonal antibodies (MAbs) and two polyclonal antisera to human recombinant interleukin 1 beta (IL-1β) and three polyclonal antisera to human recombinant interleukin 1α (IL-1α). In the secretory coil both IL-α and β were detected in the clear, but not in the dark cells. Both luminal and basal cells of the coiled and straight ducts expressed IL-1α and β, the IL-1 labelling being more intense in the luminal cells. IL-1 was not usually detected in the initial portion of the intraepidermal eccrine sweat duct, whereas intense labelling was seen in the upper part including through the stratum corneum. In skin biopsies of the palm, taken after exercise, there was only faint IL-1 labelling of the secretory cells, whereas the luminal cells of the dermal ducts showed intense labelling for both IL-1α and β. In the acrosyringium, exercise did not alter the pattern for IL-1α and β, except that in the palm, some of the antibodies to IL-1β produced a more intense immunolabelling of the acrosyringeal cells. This study identifies a distinct and similar distribution of the two forms of IL-1 throughout the eccrine sweat-gland apparatus and indicates that part of the IL-1 epidermal pool originates from the sweat.

The two major forms of interleukin 1 are IL-1α and IL-1β.[1] Recent studies have shown that extracts from normal and diseased human epidermis contain IL-1.[2–5] The source of the epidermal IL-1 is not clear. It may be synthesized by keratinocytes and cultures of keratinocytes have been found to express mRNA for IL-1α and β.[6] Western blot analysis of epidermal extracts

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has shown that the epidermis contains both the 31 kDa precursor and active 17 kDa IL-1.[3] Immunohistochemical methods have revealed both cytoplasmic and intercellular IL-1 in epidermal cells.[3,7] Other possible sources of epidermal IL-1 include production by Langerhans cells,[8] melanocytes, and leakage from dermal cells (fibroblasts, endothelial and inflammatory cells) as well as production by the sweat and sebaceous glands.

Recently it has been shown that human sweat contains both IL-1α and β as detected by Western blot, enzymoimmunoassay and biological activity.[9,10] However, the cellular source(s) of IL-1 in the sweat are not known. The secretory coil of the eccrine sweat gland is involved in the production of a precursor sweat which is isotonic or slightly hypertonic.[11,12] The secretory unit is made up of three cell types: clear (serous) and dark (mucous) cells which are involved in secretion, and myoepithelial cells which function as smooth muscle cells.[11] The clear cells secrete precursor sweat by the active transport of sodium into the lumen of the secretory coil, whereas the dark cells produce mucins, e.g. glycoproteins, which can be found along the eccrine gland lumina.[11-13] The tubuli of the eccrine glands are reabsorptive segments involved in reabsorption of sodium and chlorides, resulting in hypotonicity of the secreted sweat.[11,12] The cells of the intraepidermal part of the eccrine sweat gland (the acrosyringium) are derived from the dermal tubular cells.[14,15]

In this immunohistochemical study the cellular distribution of IL-1 in the human sweat gland apparatus was determined.

METHODS

Skin biopsies and tissue processing

Two or three millimetre punch biopsies were obtained from the skin of the back, forearm, abdomen and palm of healthy volunteers. The biopsies were snap frozen in liquid isopentane at −50°C and stored at −70°C until processed. Skin biopsies were obtained, following 30 min of exercise, from the back and palm. Cryostat sections were obtained by embedding specimens in Tissue-Tek OCT compound (4583 Miles Laboratories, Elkhart, IN, U.S.A.) at −24°C and then fixed in acetone at −20°C for 10 min. In addition, biopsy material was fixed in Bouin's fluid for 90 min and then routinely processed to provide 4 μm paraffin sections.

Histochemistry

To identify the dark and clear cells of the secretory coils some of the sections were stained with periodic acid Schiff (PAS)-diastase.

Antibodies

The antibodies used are listed in Table 1.

Immunoperoxidase labelling

The immunoperoxidase labelling was performed using the ABC method for MAb and rabbit antisera and the PAP method for sheep and goat antisera (Table 1). The sections were incubated with the respective antibodies for between 30 min and 3 h at room temperature. The sections were then incubated in the dark with 0·025% 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co., St Louis, MO, U.S.A.) and 0·003% H_2O_2 in phosphate-buffered saline (PBS) and counter-stained with haematoxylin. After being dehydrated with alcohol and cleared with xylene the sections were mounted in Permount® (Fisher Scientific, Fair Lawn, NJ, U.S.A.).

TABLE 1. Anti-interleukin 1 antibodies used for immunohistochemistry

Antibody	Species/isotype	Source*	Dilution	Immunoperoxidase method
Anti-interleukin 1β				
Cistron rabbit 02-1100	Rabbit	Cistron	1:50–1:200	ABC‡
Cistron MAb,† 02-1025	Mouse IgM	Cistron	1:36	ABC
G MAb 7	Mouse IgG2a	Glaxo	1:350	ABC
G MAb 8	Mouse IgG2a	Glaxo	1:350	ABC
Sheep S77	Sheep	Glaxo	1:50	PAP§
Anti-interleukin 1α				
Cistron rabbit 02-1150	Rabbit	Cistron	1:50	ABC
Sheep S76	Sheep	Glaxo	1:50	PAP
Goat G203	Goat	Glaxo	1:50	PAP

* Cistron Technology, Pine Brook, NJ, U.S.A.; Glaxo Institute of Molecular Biology, Geneva, Switzerland.

† MAb, monoclonal antibody.

‡ ABC, avidin–biotin complex, Vectastain ABC kit (rabbit IgG PK-4001; mouse IgG PK-4002), Vector Laboratories, Burlingame, CA, U.S.A.

§ PAP, peroxidase-goat-anti-peroxidase complexes, Dakopatts, Copenhagen, Denmark.

Controls

The following controls of immunolabelling were performed: (i) The primary anti-IL-1 antibody or antiserum was replaced by PBS or a non-relevant monoclonal antibody: anti-Leu 3a (Becton Dickinson, Mountain View, CA, U.S.A.), OKT6 (Ortho Diagnostic System, Raritan, NJ, U.S.A.), HLA-DR (Dakopatts, Copenhagen, Denmark). (ii) Tissue sections were stained for endogenous peroxidase activity without the antibody. (iii) For specific absorption 1 mg of human recombinant IL-1β was coupled to 100 μl of Sepharose beads.[3] Cistron rabbit 02-1100 (500 μl) diluted 1:200 in PBS was absorbed on the beads at 37°C for 1 h following overnight incubation at 4°C and again at 37°C for 1 h under agitation; for control of the procedure, diluted antibody was similarly processed without human recombinant IL-1. The absorbed and the non-absorbed antibodies were centrifuged at 10 000 *g* for 30 min at 4°C prior to immunoperoxidase labelling. Monoclonal antibodies to IL-1β (G MAb 8) were absorbed in a similar way except that they were diluted 1:200 in Tris-buffered saline (TBS) containing 3% bovine serum albumin (BSA) and 0·5% Nonidet P40 (LKB-Produkter, Bromma, Sweden) and that 100 μg of human recombinant IL-1β was used.

RESULTS

Interleukin 1 immunoreactivity in the dermal part of eccrine glands

All the MAb and polyclonal antisera except the Cistron rabbit 02-1100 and 02-1150 showed a similar labelling pattern in the paraffin-embedded sections (Table 2). In the cryostat sections most cells of the eccrine sweat-gland coils showed faint immunolabelling with the various antibodies that were used (Table 3). It was not possible to distinguish between the various cell

TABLE 2. Interleukin 1 immunolabelling of eccrine sweat glands from paraffin sections*

Cell type	Anti-interleukin 1β Cistron rabbit 02-1100	Sheep S77	Cistron MAb 02-1025	G MAb 7	G MAb 8	Anti-interleukin 1α Sheep S76	Goat G203	Cistron rabbit 02-1150
Secretory coil								
Clear cells	(+)	+ + +	+ + +	+ + +	+ + +	+ +	+ + +	–
Dark cells	–	–	–	–	–	–	–	–
Myoepithelial cells	–	–	–	–	–	–	–	–
Coiled and straight duct								
Cells of inner (luminal) layer	(+)	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	–
Cells of outer (basal) layer	–	– to + +	– to + +	– to + + +	– to + +	– to + + +	– to +	–
Acrosyringium								
Ductal cells of Malpighian layer	–	+	+ +	+	+ +	–	+	–
Ductal cells of stratum corneum	–	+	+	+	–	+	–	–

* Labelling: –, negative; +, weak; + +, moderate; + + +, intense; – to + +, variable.

TABLE 3. Interleukin 1 immunolabelling of eccrine sweat glands from cryostat sections*

Cell type	Anti-interleukin 1β Cistron rabbit 02-1100	Sheep S77	Cistron MAb 02-1025	G MAb 7	G MAb 8	Anti-interleukin 1α Sheep S76	Goat G203	Cistron rabbit 02-1150
Secretory coil†	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
Coiled and straight duct								
Cells of inner (luminal) layer	+ + +‡	(+)	+ +‡	+ +	+ + +‡	+ + +	+ +‡	+ +‡
Cells of outer (basal) layer	+ + +	(+)	+ +‡	+ +	(+)‡	+ +‡	+ +‡	+ +‡
Acrosyringium								
Ductal cells of Malpighian layer	+	+	+	– to + +	– to +§	+ +	+ +‡	+ + +‡
Ductal cells of stratum corneum	+	+ +	(+)	+ +	–	+ +	+ +	+ + +

* Labelling; –, negative; +, weak; + +, moderate; + + +, intense; – to + +, variable.
† Not possible to distinguish between the various cell types.
‡ Membrane-associated and intracellular staining.
§ Luminal layer only.

types in these coils. Immunolabelling was prevented by absorption of the anti-IL-1 antibodies using human recombinant IL-1 prior to staining (Figs 1 and 2).

In the secretory coil the 'clear' cells showed positive intracellular labelling, whereas 'dark' mucous cells remained unlabelled (Fig. 3). Histochemical staining of parallel sections with PAS-diastase also showed that the IL-1 immunoreactive cells were 'clear' cells. The myoepithelial cells did not show any immunolabelling. The ductal cells of both coiled and straight ducts showed intense immunolabelling (Fig. 4). In addition to intracellular IL-1, there was also membrane-associated staining for IL-1 in the tubular cells as seen in the cryostat sections (Table 3). In some ducts only the cells of the inner luminal layer were positively labelled for IL-1 and the outer layer was either only faintly positive or negative for IL-1 (Fig. 4). Intraluminal material could be detected in some cryostat sections but no immunolabelling was detected. However, some immunolabelling could be detected in the paraffin-embedded sections with some of the antibodies used.

There was only faint labelling of the secretory cells with the various antibodies but the tubular cells in the luminal layer showed intense labelling with some antibodies, e.g. G MAb 8, following exercise-induced sweating.

Interleukin 1 immunoreactivity in the acrosyringium

Compared with the dermal part of the gland, there was more variation in immunolabelling in the acrosyringium with the various antibodies used and the cryostat sections showed more intense labelling than the paraffin-embedded sections. In the cryostat sections the antibody G MAb 8, that has been shown to label mainly the basal cells of the epidermis[3] showed faint labelling of the inner cell layer of the lower part of the acrosyringium. Another antibody, G MAb 7, which showed faint labelling of the basal cell layer and a more pronounced labelling of the granular cell layer[3] did not show immunolabelling in the initial part of the acrosyringium. However, this antibody produced intense immunolabelling of the coiled duct in the stratum corneum and in the adjacent area (Fig. 5). Intense intracellular labelling for IL-1 of keratinocytes and cells of the acrosyringium occurred in the paraffin sections.

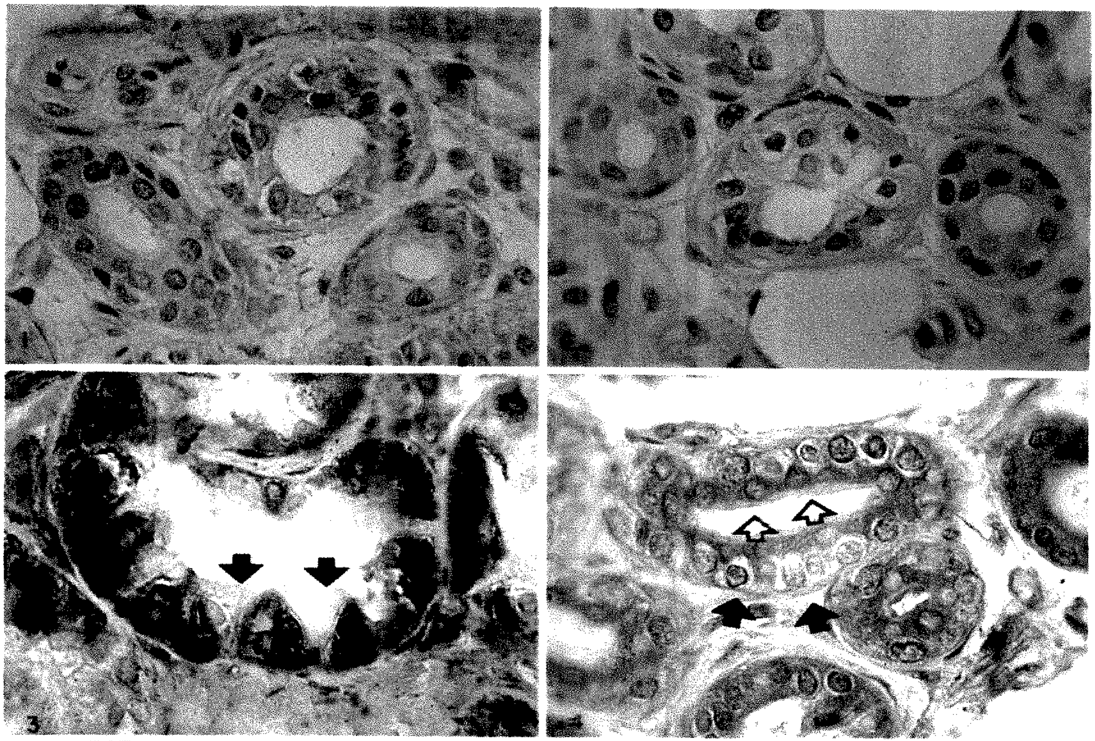
Exercise-induced sweating did not significantly modify the labelling pattern with the various antibodies. However, intense labelling for IL-1 was seen in the stratum corneum, surrounding the coiled duct, with some antibodies, e.g. G MAb 7 and Cistron rabbit 02-1100.

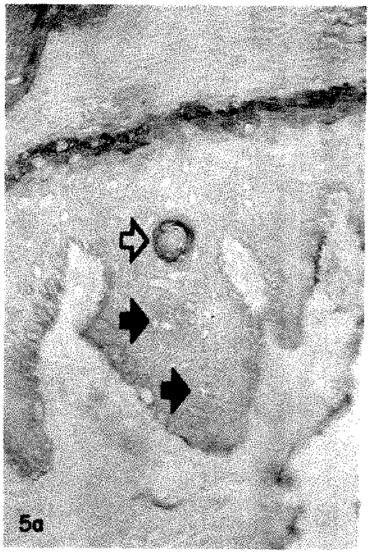
DISCUSSION

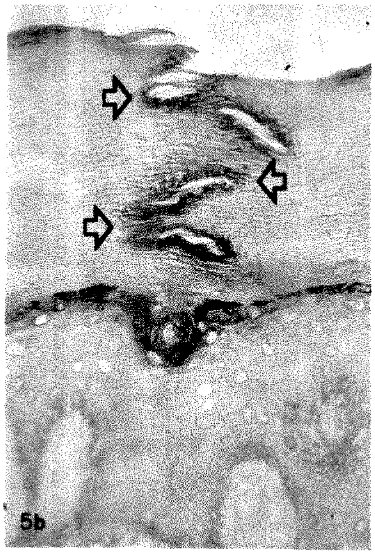
Interleukin 1α and β have previously been found in human sweat collected from several areas of the body and from the palms and soles.[9,10] IL-1α was found in all body sites, but IL-1β was only identified in sweat from the palms and soles. Western blot analysis showed that only the 17 kDa forms were detectable, indicating that secreted IL-1 accounts for the enzymoimmunoassay and biological activity instead of the 31 kDa intracellular precursor.

The collection of the sweat necessitates stimulation by heat or exercise, and it was not clear whether the resting sweat apparatus excretes IL-1. This could be determined by *in situ* hybridization with complementary DNA IL-1-specific probes that would indicate whether the sweat-gland cells not only excrete but also synthesize IL-1. This approach has so far not been feasible. Another approach is to identify material reacting with anti-IL-1 antibodies within the human eccrine sweat glands by means of immunohistochemistry.

We have studied the distribution of IL-1 in human epidermis using a panel of polyclonal and monoclonal antibodies to avoid unwanted cross-reactions in immunolabelling.[3] In the present study both IL-1α and β immunolabelling were detected throughout the human eccrine sweat gland. The labelling appears to be specific, since a panel of specific polyclonal and monoclonal antibodies raised against IL-1α and β in different laboratories yielded similar results. Absorption of two of the specific anti-IL-1 reagents with a related recombinant protein prevented immunolabelling. Therefore, unless a protein in the sweat expresses an epitope







common to both the IL-1α and β molecules, which is not likely, the staining we observed corresponds to the distribution of IL-1 within the human eccrine sweat gland.

The present results do not indicate that all the IL-1 species present in the eccrine sweat gland have been identified. There was no marked increase in IL-1 immunolabelling after exercise-induced sweating, whereas in a previous study this induced high amounts of IL-1α (and β in palms and soles) at the skin surface.[9,10] We suspect that the IL-1 immunolabelling in tissue sections corresponds to cell-associated IL-1 rather than to the intraluminal secreted species. It is well known that soluble intraluminal materials not bound to tissue structures are not usually accessible to immunohistochemistry. The total absence of IL-1 immunolabelling in cryostat sections both before and after exercise-induced sweating suggests that intraluminal IL-1 was lost during the tissue processing, whereas the positive IL-1 immunolabelling of some paraffin sections, suggests that it was at least partly preserved by the fixation and paraffin embedding.

The antibodies used reacted with both precursors and mature secreted IL-1 species.[3] However, the presence of IL-1 immunolabelling in resting (or at least not maximally stimulated) eccrine sweat glands indicates that IL-1 species are a constitutive component of human sweat and not only a stress-induced excretion, as might have been concluded from our previous studies.[9,10] Whether IL-1 species are produced by the sweat gland or simply taken up from other sources cannot be established from this study. Production by sweat-gland cells is possible because these are embryologically derived from keratinocytes, which have been shown to produce IL-1α and β.[6] The distribution of the immunolabelling along the eccrine sweat gland is also compatible with local production.

In the secretory unit, only one of the three constitutive cells (i.e. the clear cells) was found to express IL-1. We were not able to exclude the possibility of a tissue-processing artefact because the secretory units were poorly preserved in the cryostat sections. Another possibility is that IL-1 is in fact confined to the clear cells, which would indicate that a specific process (either secretion or uptake) is involved.

In the intradermal portion of the excretory duct, which is made up of the two cell layers, the IL-1 immunoreactivity was predominantly located in the inner (luminal) cell layer. The distribution of IL-1 immunolabelling at this level does not allow a distinction to be made between a process of reabsorption from the lumen or production at this level. Since the luminal

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FIGURE 1. Paraffin section of skin taken from the back and labelled with monoclonal antibody (MAb) G MAb 8. There is selective interleukin 1 immunolabelling in some cells of the secretory coil (original × 1000).

FIGURE 2. As Fig. 1 except that the monoclonal antibody (MAb) G MAb 8 was absorbed with human recombinant interleukin 1β prior to labelling. No specific labelling is seen (original × 1000).

FIGURE 3. Paraffin section of skin taken from the back and labelled with the monoclonal antibody Cistron 02-1025. Cells displaying interleukin 1 have clear cell morphology; the mucous dark cells are not labelled (arrows) (original × 1000).

FIGURE 4. Paraffin section of skin taken from the back and labelled with Goat G203. The inner (luminal) cell layer of the coiled duct displays interleukin (open arrows), whereas the outer cell layer remains largely unlabelled (closed arrows) (original × 1000).

FIGURE 5 (a) and (b) Cryostat section of the palm, labelled with the monoclonal antibody G MAb 7. Interleukin 1 is not detected at the initial part of the acrosyringium (closed arrows); intense interleukin 1 immunolabelling is, however seen in the distal part of the acrosyringium (open arrows) (original × 400).

cells showed much more intense IL-1 immunolabelling than the basal cells, the possibility of reabsorption of IL-1 appears more likely.

There was a distinctive distribution of IL-1 in the acrosyringium with the majority of the anti-IL-1 antibodies used. Very little immunolabelling was present in the initial part of the transepidermal crossing, whereas there was intense labelling above this. One possibility is that the distal cells in the acrosyringium reabsorb IL-1 from the lumen. Another possibility is that IL-1 is related to the differentiation of the cells lining the duct. We have previously[3] found that, in the epidermis, the preterminal cell in the process of differentiation of the keratinocyte, i.e. the granular cell, selectively concentrates IL-1 (see also Fig. 5). It seems that, in the acrosyringium, the distribution of granular cells also corresponds to IL-1 immunolabelling. This no longer applies to the most distal part through the stratum corneum. It may be that the immunolabelling observed corresponds here to the absorption of the excreted IL-1 into the cells, because only the 17 kDa form is detected by Western blot in human callus. Leakage from the duct in exercise-induced sweating is also compatible with this hypothesis.

To our knowledge, no previous studies have been conducted on the cellular distribution of IL-1 or other cytokines in the sweat glands. The present study establishes the presence of IL-1 throughout the human eccrine sweat gland apparatus. The distribution pattern is compatible with a dynamic process of production within several cell types involving reabsorption from the lumen in the absence of maximal stimulation and stress. The presence of both IL-1α and β is in accordance with our previous observation on sweat as collected at the surface.[9,10] The significance of the presence of the two IL-1 species in human sweat is unknown, and requires further study.

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T-cell inducer populations in cutaneous inflammation: a predominance of T helper–inducer lymphocytes (THi) in the infiltrate of inflammatory dermatoses

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SUMMARY

The mononuclear infiltrate found in a variety of inflammatory dermatoses was characterized by a predominance of T helper–inducer lymphocytes (THi), $CD4^+/CD45RA^-/CD45RO^+$, a population of cells responsible for maintaining and promoting immune reactions. Only small numbers of T-suppressor–inducer lymphocytes (TSi), $CD4^+/CD45RA^+/CD45RO^-$, cells responsible for inducing CD8 suppressor–effector cells to 'down regulate' immune reactions, were seen. The predominance of $CD4^+$ THi lymphocytes was common to all dermatoses studied and suggests a common final pathway in chronic cutaneous inflammation, irrespective of initial causative factors.

The identification *in vitro* of function-related antigens on immunologically competent cells such as lymphocytes, has produced significant advances in the understanding of immunological mechanisms *in vivo*. The initial demonstration of two classes of lymphocytes, B and T cells,[1] and the later identification of subset-specific cell-surface antigens[2] allowed T cells to be divided into T-helper (T_h) cells responsible for helper and delayed-type hypersensitivity reactions and T-suppressor (T_s) cells responsible for cytotoxic and suppressor functions. The infiltrates associated with a wide variety of dermatoses characteristically show a predominance of T_h cells,[3] suggesting an important role for these cells in cutaneous inflammation.

Recently, a number of monoclonal antibodies have been developed which establish a close correlation between the specific immunoregulatory function of lymphocytes and their phenotype. Using such antibodies, $CD4^+$ T_h lymphocytes can now be divided into two distinct groups with differing regulatory and effector roles, namely helper inducer (THi) cells and

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suppressor inducer (TSi) cells. CD4$^+$ THi cells, identified by the antibodies 4B4[4] and UCHL1,[5] respond preferentially to soluble antigen and provide help for B-cell immunoglobulin synthesis. CD4$^+$ TSi cells, identified by the antibodies 2H4,[6] Leu 8[7] and Leu 18,[8] proliferate in response to mitogen and autologous mixed lymphocyte reactions (AMLR) and induce CD8 suppressor–effector cells to down regulate immune reactions. Both populations induce CD8 cytotoxic effector cells (Table 1).

To understand more fully the immunological mechanisms regulating cutaneous inflammation, it is important to establish the presence and relative proportions of CD4$^+$ inducer subsets within the predominant CD4$^+$ infiltrate found in the inflammatory dermatoses. The distribution of helper inducer and suppressor inducer regulatory subsets was studied and a double-labelling immunohistochemical technique was developed that allowed *in situ* demonstration of colocalized, inducer-specific antibodies to CD4$^+$ cells in skin sections.

METHODS

Skin samples

An ellipse of skin was removed under local anaesthetic for diagnostic purposes; half was routinely processed for histopathological assessment, the other half was snap frozen in liquid nitrogen-cooled isopentane using OCT cryoprotectant (Tissue Tec, Miles, U.S.A.) and stored at −70°C until used. Specimens included atopic dermatitis (6), contact dermatitis (6), psoriasis (7), lichen planus (6) and discoid lupus erythematosus (4).

Double-labelling immunohistochemical reaction

To colocalize inducer-specific antibodies to CD4$^+$ lymphocytes, an amplified three-step double-labelling system was developed based on the ability of 3,3′-diaminobenzidene (DAB), when developed in excess, to immunologically seal the first antibody complex from cross-reactions with antibodies used in demonstrating the second antigen.[9] Cryostat sections (5 μm) were cut and air-dried (10 min) prior to fixation in acetone (5 min). Following incubation with normal swine serum (10 min), sections were labelled with the CD4 marker, Leu 3a (1:10 dilution, 60 min). Subsequent incubation with a peroxidase-conjugated rabbit anti-mouse antibody (1:10 dilution, 30 min) was followed by reaction with mouse peroxidase anti-peroxidase (PAP) complexes (1:20, 30 min). Peroxidase was visualized using DAB (10 min

TABLE 1. Characteristics of CD4$^+$ inducer subsets

	Helper–inducer	Suppressor–inducer
	CD4$^+$ UCHL1$^+$ 4B4$^+$	CD4$^+$/Leu 18$^+$/Leu 8$^+$/2H4$^+$
Proliferation to soluble antigen	+	−
Proliferation to mitogens	−	+
Proliferation in AMLR	−	+
Help for B cell Ig production	+	−
Induction of CD8 lymphocyte-mediated suppression	−	+
Induction of CD8 lymphocyte-mediated cytotoxicity	+	+

incubation in darkness). The sections were then incubated with inducer-specific second primary antibodies (see Table 2 for relevant dilutions) for 60 min, prior to applying an alkaline-phosphatase-conjugated rabbit anti-mouse bridging antibody (1 : 10 dilution, 30 min) followed by mouse alkaline-phosphatase anti-alkaline-phosphatase (APAAP) complexes (1 : 20 dilution, 30 min). The second antigen was visualized using fast blue as substrate, with the development reaction being in two stages of 30 min each separated by buffer washing. Sections were counterstained with Mayers haemalum and mounted in Uvinert (BDH Ltd., Poole, Dorset, U.K.), a permanent water-soluble mounting medium, prior to visualization. Antibody reactions were separated by 10 min washes with Tris-buffered saline throughout. All secondary and tertiary antibodies were supplied by Dako Ltd. (High Wycombe, Bucks, U.K.). Slides were coated with 3-aminopropyltriethoxysilane (Sigma Chemical Co., St Louis, MO, U.S.A.) which produces an exceptionally strong bond between section and slide allowing rigorous washing to remove reagents between the multiple steps.[10]

The use of conjugated secondary antibodies in both the PAP and APAAP reactions improved the contrast of positively-labelled cells without loss of sensitivity. Fast blue proved to be the most useful substrate for the APAAP reaction, as the combination of colours seen in the final reaction, i.e. single-labelled brown or blue lymphocytes, were clearly distinguished from the grey/black reaction product of double-labelled, CD4$^+$ THi or TSi lymphocytes (Fig. 1).

Staining combinations

Cryostat sections (5 μm) were stained as above using the CD4 marker Leu 3a in the initial PAP reaction, followed by APAAP reactions with 4B4, UCHL1, Leu 8, Leu 18, 2H4 and Leu 2a on serial sections (see Table 2 for antibody reactivities and optimal dilutions).

Controls

To ensure no cross-reaction was occurring during double-labelling, parallel staining was performed on a control section using the non-colocalizing combination of OKT6 and Leu 1 antibodies. Single staining reactions using Leu 3a (PAP) and Leu 2a, 2H4, Leu 8, Leu 18, 4B4 and UCHL1 (APAAP) were performed on tonsil and each skin specimen as a positive control for each antibody. Negative controls included omission of primary antibodies, substitution by

TABLE 2. Characteristics of primary antibodies used

			T cell reactivity %			
Antibody	Antigen	Dilution	CD3	CD4	CD8	Non-T lineage reactivity
Leu 3a*	CD4	1 : 10	45	100	—	Monocytes, macrophages
Leu 2a*	CD8	1 : 30	30	—	100	NK cells
UCHL1† (THi)§	CD45RO	1 : 30	40	70	35	Granulocytes, monocytes
4B4‡ (THi)§	CDW29	1 : 400	41	41	43	Some B cells, null cells, macrophages
2H4‡ (TSi)§	CD45RA	1 : 50	42	41	54	B cells, null cells
Leu 8* (TSi)§	p80	1 : 10	69	76	58	B cells, granulocytes, monocytes, NK cells
Leu 18* (TSi)§	CD45RA	1 : 20	40	50	70	B cells, null cells

* Beckton Dickinson, Mountain View, CA, U.S.A.
† Dakopatts, Glostrup, Denmark.
‡ Coulter Immunology, Hialeah, FL, U.S.A.
§ When colocalized with CD4 marker.

irrelevant monoclonal antibodies of similar isotype, omission of second and third step reagents and reactions with DAB and fast blue alone.

Quantification

Using an eyepiece graticule orientated along the dermo-epidermal junction, sequential fields were studied at × 1000 (oil emersion) and the number of single- (blue or brown) and double- (grey-black) labelled cells was noted until a total of 800 mononuclear cells had been recorded. Two slides of each double-staining combination were counted, the means calculated and used for analysis. Duplicate counts on coded slides were performed and compared using linear regression analysis ($r = 0{\cdot}85$) confirming the reproducibility of the counting technique.

RESULTS

Antibody reactivities

Demonstration of T-helper-inducer lymphocytes. The antibody 4B4 (CDw29) gave poor technical results in frozen skin. Despite titration of all reagents, 4B4 continued to cross-react with basal cells in the epidermis and endothelial cells of superficial vessels with substantial background staining of the dermis. Localization of substrate around individual lymphocytes was poor, making it unsuitable for double-labelling reactions or quantitative single-labelled studies. However, UCHL1 (CD45RO) produced strong, well localized reproducible results with no such cross-reactions in skin sections. The combination of Leu 3a/UCHL1 was therefore used to indicate THi cells in quantitative double-labelling studies.

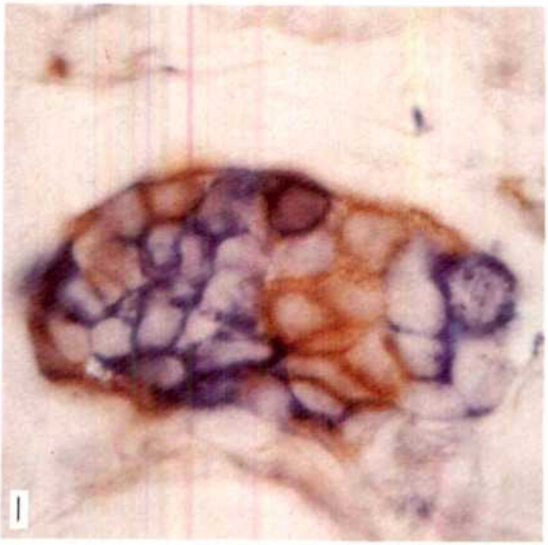
Demonstration of T-suppressor-inducer lymphocytes. Both 2H4 (CD45RA) and Leu 8 gave membrane staining of lymphocytes, although their reaction products in spite of amplification were substantially weaker and less consistent than those of Leu 18 (CD45RA). Leu 8 which gave particularly variable staining results is known to be an unstable antigen sensitive to cold-induced damage, with its expression on lymphocytes being reduced or lost following cryopreservation.[11] Whilst single-staining reactions with these antibodies were sometimes acceptable, only the combination of Leu 3a/Leu 18 was felt to be sufficiently reproducible for meaningful quantitative analysis of TSi cells in frozen skin sections.

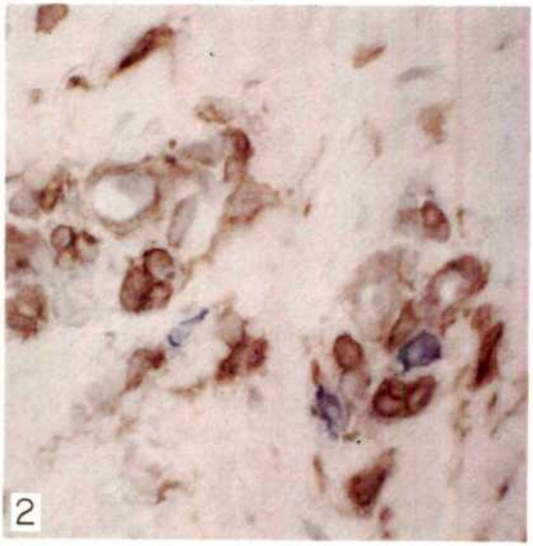
Single-staining reactions

2H4, Leu 8 and Leu 18 positive lymphocytes were substantially reduced (< 15%) in the infiltrates of the inflammatory dermatoses studied when compared to findings in normal peripheral blood (40–70%)[6–8] The only exception was seen in contact dermatitis biopsies at 96 h, where > 50% of the infiltrating mononuclear cells (predominantly $CD4^+$) were Leu 8^+. Serial sections showed these cells to be negative for 2H4 and Leu 18. In the majority of dermatoses studied more than 50% of the infiltrate showed positive staining with UCHL1.

CD4 inducer subset findings

In all dermatoses studied, only occasional dermal lymphocytes of the $CD4^+/CD45RA^+$ (Leu 18) TSi phenotype were detected (Fig. 2). In contrast however, a large number of $CD4^+$ lymphocytes showed the double-staining phenotype $CD4^+/UCHL1^+$ (Fig. 3), indicating that dermal THi cells constitute the majority of inducer cells in the infiltrates of chronic inflammatory dermatoses (Table 3). Whilst THi lymphocytes always formed the majority of the





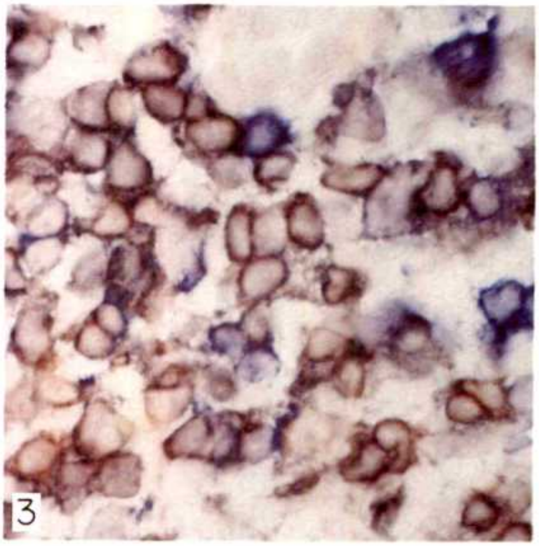


FIGURE 1. Reliable reproducible three-colour staining of frozen skin sections, showing within a single vessel Leu3$^+$ (brown), UCHL1$^+$ (blue) and a single Leu3$^+$/UCHL1$^+$ double-labelled T helper-inducer lymphocyte (grey black) (× 320).

FIGURE 2. Atopic dermatitis. Double-staining reaction revealing Leu 3 (brown) and Leu 18 (blue) positive cells. Note the paucity of Leu 18 cells, and that those present are not double-stained and, therefore, do not represent T suppressor–inducer lymphocytes (× 80).

FIGURE 3. Lichen planus. Double staining combination of Leu 3 (brown) with UCHL1 (blue). Note that whilst occasional single stained cells can be seen the majority of lymphocytes double-stain and hence have the Leu 3$^+$/UCHL1$^+$ (grey–black) phenotype indicative of T helper–inducer cells (× 160).

TABLE 3. The presence of THi and TSi cells within the CD4 population of lymphocytes in inflammatory dermatoses (mean %, of CD4$^+$ cells ± SEM)

	CD4:CD8	THi (Leu 3/UCHL1)	TSi (Leu 3/Leu 18)
Atopic dermatitis (6)	8:1	63 ± 8	1 ± 1
Contact dermatitis (6)	4:1	48 ± 6	3 ± 2
Lichen planus (6)	8:1	69 ± 8	6 ± 3
Psoriasis (7)	9:1	42 ± 6	10 ± 6
Discoid lupus erythematosus (4)	8:1	40 ± 15	7 ± 3

lymphocyte population, the ratios of THi:TSi varied from 4:1 (psoriasis) to 63:1 (atopic dermatitis).

Summation of the THi and TSi populations does not reach 100%, implying that there are a substantial number of CD4$^+$ lymphocytes in the skin without either inducer phenotype. This is a similar situation to that found in the peripheral blood, where 20–30% of circulating CD4$^+$ lymphocytes carry no inducer markers.[6]

DISCUSSION

Three main conclusions arise from this study. Firstly, there is a predominance of helper–inducer lymphocytes (Leu 3a$^+$/UCHL1$^+$) found in the infiltrates associated with chronic inflammatory dermatoses. Secondly, very few suppressor–inducer lymphocytes (Leu 3a$^+$/Leu 18$^+$) are present at the site of inflammation. Thirdly, the above findings are not disease specific, and are found in a range of different inflammatory dermatoses. The phenotype of the predominant cell type is, therefore, that of a population whose functional role is in keeping with ongoing inflammation. Furthermore, whilst relative numbers do not necessarily correlate with functional ability, a shortage of TSi cells would lead to minimal induction of CD8 suppressor–effector cells with a subsequent weakening of the negative arm of the immune response. These findings implicate disordered inducer subsets in maintaining inflammatory pathways in cutaneous disease.

This quantitative study extends the findings of previous work[12,13] and demonstrated a predominantly THi population with few TSi cells in the infiltrate associated with inflammatory dermatoses. Recently, an apparently contradictory report[14] described 'significant' numbers (not quantified) of CD45RA$^+$ (2H4$^+$) cells in normal skin. However, double-staining reactions with CD4 markers were not performed, and as CD45RA antibodies bind to both CD4 and CD8 lymphocytes, interpretation of single-labelled cells as TSi lymphocytes is not possible. Modlin *et al.*[15,16] using 2H4, 4B4 and Leu 3a antibodies demonstrated a 14-fold increase of THi over TSi cells in tuberculoid leprosy lesions, changing to a 1:1 ratio in lepromatous leprosy. Such a change demonstrates that phenotypically defined inducer populations may reflect functional immunological mechanisms *in vivo*. Whilst the predominance of THi over TSi lymphocytes was seen in all the chronic inflammatory dermatoses studied, suggesting a final common pathway in dermal inflammation irrespective of the initial aetiological cause, the variability in ratios of THi:TSi seen is interesting. In particular, atopic dermatitis seems to be characterized by a very marked paucity of TSi when compared with the other dermatoses studied.

In the peripheral blood, the ratio between circulating THi and TSi is approximately 1·2–1.[17] The predominance of THi lymphocytes seen in the dermatoses studied, therefore, implies a mechanism by which differential subset distribution is seen at sites of dermal inflammation. One possible mechanism would be a preferential uptake of soluble antigen-activated (THi) lymphocytes from the circulation into the skin. As the initial migration of lymphocytes into cutaneous tissue involves the adhesion of such cells to endothelium, it is of interest that $CD4^+/CDw29^+(4B4^+)$ THi cells are more adherent than TSi cells in a lymphocyte–endothelial binding assay.[18] Alternatively, a selective *in situ* proliferation of THi cells at the site of antigenic challenge would lead to their predominance in cutaneous inflammation. However, this seems unlikely, as our own studies[19] with the Ki67 monoclonal antibody show very few T cells to be actively proliferating. A further possible mechanism is explained by the recent suggestion that $CD4^+/CD45RA^+/UCHL1^-$ lymphocytes represent immature, virgin T cells, and that such lymphocytes express the UCHL1 antigen when they differentiate into active memory T cells.[20] Thus, dermal CD4 lymphocytes may have acquired the UCHL1 antigen as a result of differentiation in response to an *in situ* antigenic stimulus.

Our findings suggest that in a number of dermatoses characterized by ongoing chronic inflammation, a preponderance of THi cells with a paucity of TSi cells is found. This pattern is similar to findings in chronic inflammatory arthropathies,[21] and pleural and peritoneal inflammatory infiltrates,[18] suggesting that it represents a general manifestation of chronic inflammation and not a skin-specific reaction pattern. Furthermore, the clinical differences between the various inflammatory dermatoses cannot be explained by our present knowledge of T-inducer lymphocyte function.

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Differential modulation of keratinocyte intercellular adhesion molecule-1 expression by gamma interferon and phorbol ester: evidence for involvement of protein kinase C signal transduction

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SUMMARY

There is growing evidence that keratinocyte (KC) intercellular adhesion molecule-1 (ICAM-1) expression is involved in the epidermal trafficking of T lymphocytes. To further characterize the molecular basis of KC ICAM-1 expression, the detailed kinetics of induction by gamma interferon (IFN-γ), as well as the phorbol ester, 12-O tetradecanoylphorbol-13-acetate (TPA), were studied. This study reports that KCs express both the class II major histocompatibility antigen (HLA-DR) and ICAM-1 in response to IFN-γ, although the response is distinctive for each molecule. Also, TPA induces ICAM-1, but not HLA-DR expression, whilst the protein kinase inhibitor, H7, blocks the TPA, but not the IFN-γ-mediated response. The results provide a molecular basis whereby non-cytokine-mediated stimuli (e.g. TPA) alter KC signal transduction events involving protein kinase-C (PK-C) and thereby generate such immunologically relevant events as ICAM-1 expression. Thus, KCs may be targets for both T-cell derived cytokines (e.g. IFN-γ), and non-cytokine TPA-like molecules which stimulate PK-C. Induction of ICAM-1 by either mechanism would be capable of instigating intraepidermal T-cell trafficking.

There is evidence implicating keratinocyte (KC) intercellular adhesion molecule-1 (ICAM-1) expression in the epidermal trafficking of T lymphocytes.[1-6] Recently, we have reported on the ability of gamma interferon (IFN-γ) to induce different patterns of KC ICAM-1 and HLA-DR expression.[2] T cells which express lymphocyte-function-associated antigen-1 (LFA-1) bind to

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ICAM-1-expressing KCs *in vitro*,[7-9] and the *in vivo* appearance and localization of intraepidermal T cells associated with KC ICAM-1 expression has been correlated to the induction, maintenance, and resolution phases of a wide variety of skin diseases.[3-6,10,11] Thus, the regulation of KC ICAM-1 expression is likely to be important in our understanding of the mechanism of cutaneous inflammatory reactions. In psoriasis, for example, KC ICAM-1 expression[3-6] is accompanied by increased levels of diacylglycerol (DAG) with activation of its target molecule, PK-C.[12-13] Furthermore, topical application to mice of TPA and DAG causes marked inflammation and increased epidermal proliferation which simulates the hyperplastic response as seen in psoriasis.[14] To determine whether altered PK-C signal transduction could directly influence ICAM-1 expression, cultured KCs were exposed to both TPA, a potent PK-C agonist, and a known ICAM-1 inducer (IFN-γ).[2,5,6,9] The subsequent expression of ICAM-1 was measured with the use of immunoperoxidase staining techniques and fluorescence-activated cell sorting (FACS).

METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). Serum free, KC growth medium containing low calcium (KGM) was obtained from Clonetics Co., (San Diego, CA, U.S.A.). Recombinant IFN-γ (specific activity = $1{\cdot}7 \times 10^7$ U/mg) was provided by Dr M. Shepard, Genentech, Inc., (South San Francisco, CA, U.S.A.). Calcium ionophore (A23187), digitonin, TPA, EDTA, EGTA, Tris, gum tragacanth, and mercaptoethanol (β-ME) were purchased from Sigma Chemical Co., (St Louis, MO, U.S.A.). (^{32}P) deoxycytidine triphosphate was from ICN Radiochemicals (Irvine, CA, U.S.A.) DEAE cellulose (DE52) was obtained from Whatman (Hilsboro, OR, U.S.A.), protein kinase-C inhibitor H7[15] was obtained from Seikagaku Kogyo Co. (Tokyo, Japan). All other chemicals were of at least reagent grade.

KC culture

Samples of normal, human skin obtained from cosmetic surgery were processed to single cell suspensions and viable KCs were then seeded on to, and grown in either 35-mm diameter, plastic Petri dishes (Lux. Flow Laboratories Inc., Naperville, IL, U.S.A.) or Lab-Tek chamber slides (Miles LAB, Naperville, IL, U.S.A.) in KGM as previously described.[2] Cells were maintained in a humidified incubator in 5% CO_2/95% air at 37°C and used after passage 3.

TPA induction of KC expression of ICAM-1 and HLA-DR

TPA, A23187 and H-7 were added to subconfluent KCs growing in KGM in the indicated combinations, concentrations and time courses. KCs grown in Petri dishes were then trypsinized, stained, and examined by FACS analysis. KCs grown in Lab-Tek chamber slides were stained *in situ* using an immunoperoxidase technique.

Staining of KCs for ICAM-1 and HLA-DR

KCs grown in 35-mm dishes were trypsinized using 0·03% trypsin/0·01% EDTA and an aliquot of approximately 10^6 KCs incubated for 40 min on ice with 10 μg/ml of the primary monoclonal antibodies, anti-ICAM-1 (RR 1/1; Dr T. Springer, Boston, MA, U.S.A.) and anti-HLA-DR (TS 1·16; Dr C. Clayberger, Stanford, CA, U.S.A.). Anti-Leu 2a (CD8, Becton-Dickinson, CA, U.S.A.) was used as an isotype control antibody. The cells were washed and then stained with 1 μg of fluoroscein isothiocyanate-conjugated (FITC) goat anti-mouse IgG (Tago, Burlingame, CA, U.S.A.) for 30 min. After washing, the stained cells were fixed in 2% paraformaldehyde and staining intensity and numbers of cells stained, determined by FACS (EPICS 541, Coulter Corp.) as previously described.[2] KCs grown in Lab-Tek chamber slides were stained *in situ* with the aforementioned monoclonal antibodies using an immunoperoxidase technique (Vectastain ABC Kit, Vector laboratories, Burlingame, CA, U.S.A.) with 3-amino-9-ethylcarbazole as the chromogen and counterstained with 1% haematoxylin.[3]

Protein kinase-C activity

Gamma interferon, at 10^3 U/ml, was added to 100-mm diameter Petri dishes containing subconfluent KCs for either 15, 30 or 60 min. TPA 100 nM was added to similar dishes for 10 min to serve as a control. At the indicated times, the treated dishes were washed with cold, phosphate-buffered saline (PBS) (calcium and magnesium free) and then incubated on ice for 5 min in the presence of 3 ml of permeabilizing buffer (20 mM Tris, 5 mM EGTA, 2 mM EDTA, 10 mM β-ME, Leupeptin and Digitonin).[16] The permeabilizing buffer was collected (soluble fraction) and kept on ice whilst the KCs were harvested in 3 ml of homogenizing buffer (20 mM Tris, 5 mM EGTA, 2 mM EDTA, 10 mM βME, Leupeptin and 0·5% Triton X-100) and centrifuged at 10 000 ***g*** for 40 min, the resulting supernatant being the membrane fraction. Both soluble and membrane-associated PK-C were partially purified by DEAE cellulose chromatography and the fractions assayed in duplicate for PK-C activity by measuring the incorporation of ^{32}P from ^{32}P-ATP into histone as described.[17] Protein estimation was performed using the Bio-Rad assay.[18]

Time course and dose-dependence of IFN-γ induced KC expression of ICAM-1 and HLA-DR

IFN-γ was added to subconfluent, KC monolayers in 35-mm dishes at the indicated concentrations and incubated with the KCs for the various time courses. The KCs were then trypsinized, stained and quantified by FACS analysis, or the IFN-γ was washed from the dishes and the KCs incubated further in KGM alone for the indicated periods of time until trypsinization and staining.

RESULTS

Induction of KC ICAM-1 expression by TPA

By FACS analysis, TPA induced KC expression of ICAM-1 but no HLA-DR and the maximal induction occurred at 1 day with no subsequent increase or decrease in intensity of expression with incubation continued for as long as 3 days. Incubation beyond 3 days resulted in TPA-induced KC toxicity. The maximum dose response was reached at 10–20 nM TPA (Fig. 1). Neither A23187 nor the protein kinase inhibitor, H-7 alone induced ICAM-1 or HLA-DR expression, but A23187 at 1 μM enhanced TPA-induced expression of ICAM-1. H-7 at 40 μM inhibited TPA induction of ICAM-1 by 50% (higher H-7 concentrations could not be used due to cytotoxicity), and TPA + A23187 induction of ICAM-1 by 60% (Fig. 2), but did not inhibit IFN-γ-induced ICAM-1 expression (data not shown).

Immunoperoxidase staining of untreated KC monolayers showed few KCs (< 1%) positive for ICAM-1. However ICAM-1, but not HLA-DR expression, occurred after incubation for 48 h with TPA alone, or with TPA and A23187, although the intensity and extent of ICAM-1 expression was less than that induced by IFN-γ (Fig. 3). Light microscopy showed that those

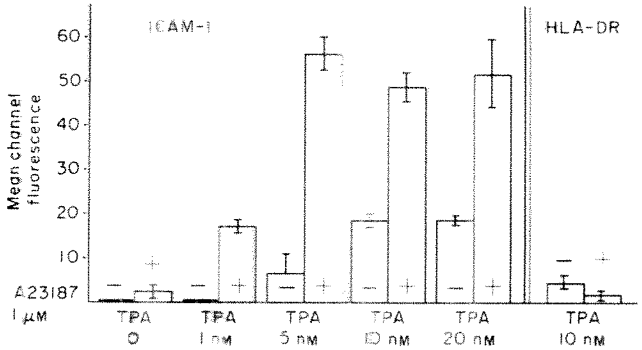

FIGURE 1. KC expression of ICAM-1 and HLA-DR following incubation for 48 h with TPA 0–20 nM ± calcium ionophore, A23187, 1 μM, as determined by FACS analysis. A23187 enhances the induction of ICAM-1 by TPA, and there is only minimal HLA-DR expression in response to TPA with no enhancement by A23187. ($n = 3$–7 for all experiments, values are mean ± SEM.)

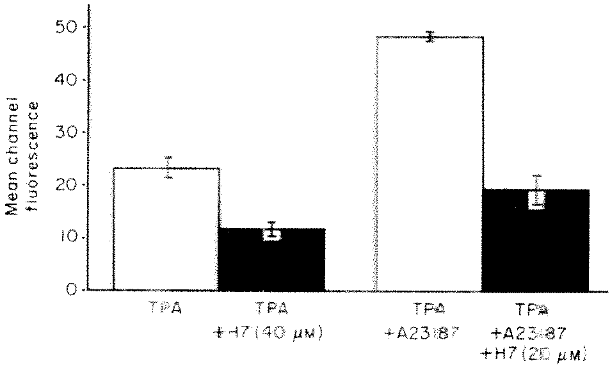

FIGURE 2. KC expression of ICAM-1 after 48 h incubation with either TPA 10 nM or TPA 10 nM + calcium ionophore, A23187, 1 μM and subsequent inhibition of this effect by co-incubation with PK-C inhibitor H7 at either 40 or 20 μM. FACS analysis determination. ($n = 4$, values are mean ± SEM.)

KCs expressing ICAM-1 in response to TPA differed from surrounding non-ICAM-1-expressing cells in that they were slightly elongated and had migrated above the monolayer.

PK-C modulation

TPA produced a rapid (10 min) reduction in soluble fraction PK-C activity (Table 1) with a concomitant rise in membrane-associated PK-C activity, as previously reported.[19,20] IFN-γ had induced no change in membrane-associated PK-C activity by 15 min. However, extension of the IFN-γ co-incubation time to 30 min produced a rise in the PK-C activity of both the membrane-associated and soluble fractions with the membrane-associated PK-C activity returning to the original value at 60 min.

Modulation of KC ICAM-1 by IFN-γ

FACS analysis demonstrated that in the absence of IFN-γ there was no KC expression of HLA-DR and < 1% staining for ICAM-1. After only 4 h of IFN-γ treatment, KCs strongly expressed

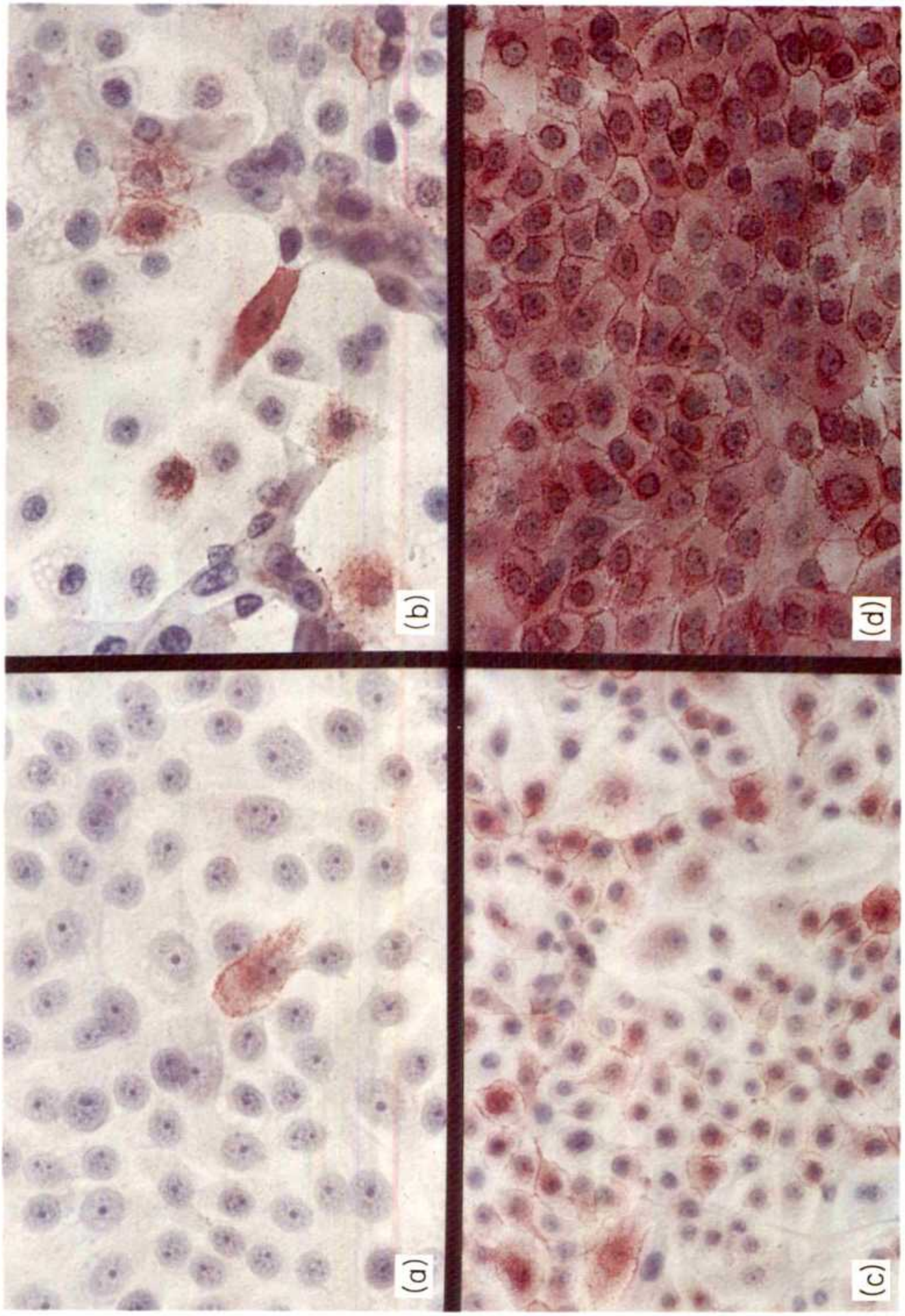

FIGURE 3. (a) Immunohistochemical ICAM-1 staining of KCs in the absence of any treatment or (b) presence of TPA alone, (c) TPA + A23187, and (d) IFN-γ after 2 days. (×75).

TABLE 1. PK-C activity, soluble and membrane-associated, of normal, human cultured KC showing effect of IFN-γ (100 U/ml) and TPA (100 nM) at different time intervals. Values are mean ± SEM

Treatment	PK-C activity (pmol/mg protein per min)	
	Soluble	Membrane associated
Control	23·1 ± 7 (*n* = 3)	43·9 ± 8 (*n* = 4)
TPA 10 min	13·4 ± 7·6 (*n* = 3)	138·9 ± 16·3 (*n* = 3)
IFN-γ 15 min	42·3 ± 12 (*n* = 3)	35·8 ± 8 (*n* = 4)
IFN-γ 30 min	35·8 ± 16 (*n* = 3)	71·3 ± 12 (*n* = 3)
IFN-γ 60 min	35·5 ± 12 (*n* = 3)	43·7 ± 12 (*n* = 3)

ICAM-1, reaching a maximum intensity at 2 days (Fig. 4). HLA-DR was not induced until day 1, with a maximum at day 3 as previously described.[21] Continued incubation with IFN-γ over a period of 6 days resulted in a marked decline of ICAM-1 expression, but a stabilizing of HLA-DR expression with minimal loss of intensity (Fig. 4). When the KCs were washed free of IFN-γ (as detected by a sensitive radioimmunoassay; Centocour, Malvern, PA, U.S.A.) after 2 days exposure, both HLA-DR and ICAM-1 expression declined slightly over the first week with a greater decrease during week 2 (Fig. 5). The decrease in HLA-DR expression was always less than for ICAM-1 to day 11, but by day 18 there was no significant detectable KC expression of either antigen.

Minimal KC expression of ICAM-1 was induced by only 0·1 U/ml IFN-γ with a rapid increase in intensity of expression with concentrations up to 5 U/ml IFN-γ (Fig. 6). Maximum expression of ICAM-1 occurred at about 10^2 U/ml IFN-γ, with increasing concentrations up to 10^3 U/ml IFN-γ producing no further enhancement. KC expression of HLA-DR was less sensitive to IFN-γ than ICAM-1, with minimal expression first occurring at 2·5 U/ml with a maximum at 100 U/ml, equivalent to that seen for ICAM-1 (Fig. 6). Thus, ICAM-1 induction in cultured KCs is more rapid and more sensitive to lower doses of IFN-γ than is HLA-DR, as was also found with short-term, whole-skin organ cultures.[3] Whether the decreased KC

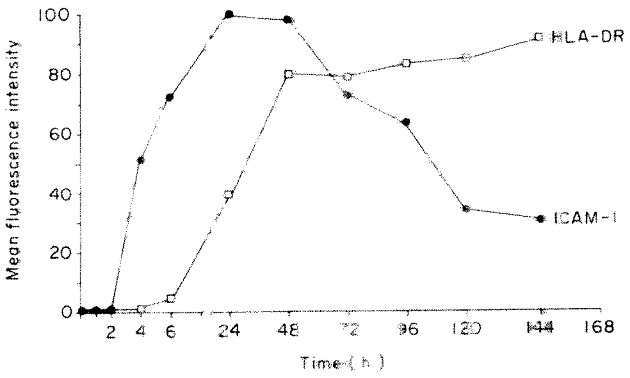

FIGURE 4. Time course of induction of ICAM-1 and HLA-DR on KCs by IFN-γ 500 U/ml for 1–144 h, as determined by FACS analysis. Note the more rapid induction and disappearance of ICAM-1 expression compared to HLA-DR.

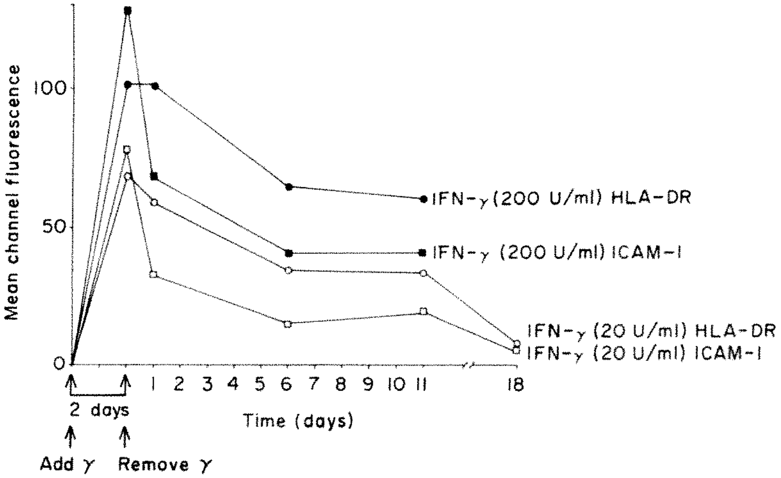

FIGURE 5. KC expression of ICAM-1 and HLA-DR after incubation with IFN-γ (20 U/ml or 200 U/ml) for 48 h, followed by washing KCs free of IFN-γ and incubation continued in KGM alone for a further 16 days by FACS analysis. Note more rapid induction of ICAM-1 expression and gradual decline in both ICAM-1 and HLA-DR expression after removal of IFN-γ.

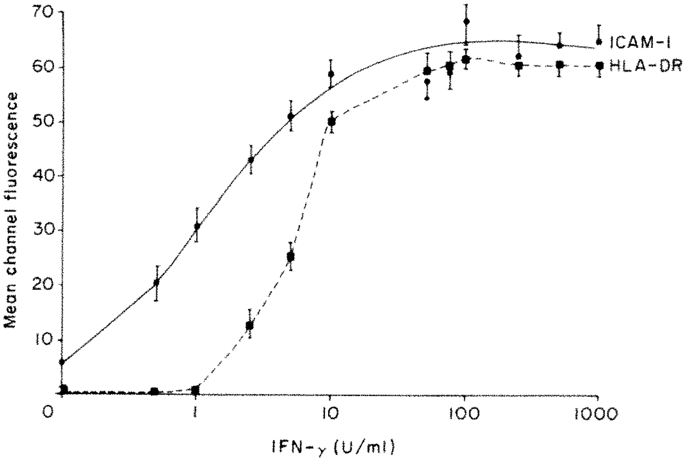

FIGURE 6. KC expression of HLA-DR and ICAM-1 in response to 48 h incubation with increasing concentrations of IFN-γ (0·5–1000 U/ml), as determined by FACS analysis. KC expression of ICAM-1 is more sensitive to lower concentrations of IFN-γ, and both KC ICAM-1 and HLA-DR expression peak at 100 U/ml of IFN-γ.

ICAM-1 expression which occurred between 48–96 h of IFN-γ exposure was due to ICAM-1 shedding, surface modification, or internalization and degradation is now being investigated.

DISCUSSION

Protein kinase C (PK-C) is the major cellular phorbol-ester receptor and it is believed that most TPA-induced events within the cell are PK-C mediated.[22] TPA induces KC ICAM-1, and this effect is enhanced by the calcium ionophore, A23187, a carboxylic acid antibiotic which transports calcium ions across biological membranes.[23] These findings strongly indicate a role for PK-C in the secondary signalling mechanisms preceding ICAM-1 expression, which is further supported by the ability of the protein kinase inhibitor, H7, to partially block this response.[15]

The observation that 15 min incubation with IFN-γ did not increase the PK-C activity of membrane-associated fractions of human KCs is in contrast to the rapid TPA activation of PK-C by translocation from cytosol to membrane.[22] However, IFN-γ increased both membrane-associated and soluble PK-C activity within 30 min. In agreement with our results, Hamilton *et al.*[24] demonstrated a concomitant increase in both cytosolic and membrane-associated PK-C activity following treatment of murine peritoneal macrophages with IFN-γ. They concluded that this dual rise in soluble and membrane PK-C activation was not due to *de novo* synthesis of PK-C and that the molecular mechanisms were unknown. Ostrowski *et al.*[25] showed in a B-lymphoid cell line that although IFN-γ will produce transient translocation of PK-C activity from cytoplasm to membrane, the response is slower (30 min) than that reported for interleukin 3.[26]

Our study therefore agrees with previous work which demonstrated that IFN-γ does not activate PK-C in the same manner as does TPA. The inability of H7 to inhibit IFN-γ-induction of KC ICAM-1 is evidence that IFN-γ does not directly induce KC expression of ICAM-1 via a PK-C-mediated pathway. Furthermore, Koide *et al*[27] demonstrated that IFN-γ induction of HLA-DR on HL-60 cells is not inhibited by H7, but W7 (a calmodulin antagonist) will block this response, indicating that IFN-γ induction of HLA-DR may be a calcium/calmodulin and not a PK-C-mediated event. Perhaps IFN-γ induction of KC ICAM-1 expression is also via a calmodulin-dependent pathway. Previous authors have observed that certain biological effects of IFN-γ, such as induction of an anti-viral state, are PK-C dependent,[28] whilst other activation events are PK-C independent.[29]

The results suggest that KC ICAM-1 is inducible by at least two different cellular mechanisms; the first involving IFN-γ, and the second involving TPA. In the first mechanism the KCs may be viewed as passive targets for IFN-γ which can augment an ongoing immunological reaction in the dermis and which induces ICAM-1 expression. In the second mechanism, the KCs may be viewed as active initiators of epidermal T-cell trafficking in which PK-C-mediated induction of ICAM-1 leads to the activation of T cells and subsequent production of T-cell cytokines. In this model, either endogenously generated DAG within the epidermis, or exogenously applied TPA-like molecules to the skin surface, would be responsible for activation of KC PK-C and induction of KC ICAM-1 expression.

It is known that even normal appearing skin contains occasional intraepidermal T cells,[30] and accumulation of these T cells within the epidermis occurs when they bind to KCs expressing ICAM-1. These accumulated intraepidermal T cells may become activated themselves either through exposure to KC-derived cytokines such as IL-1,6, GM-CSF,[31] or through activation of their own PK-C signal transduction system by a TPA-like molecule.[32] Activated intraepidermal T cells could then produce IFN-γ to augment KC ICAM-1 expression and induce HLA-DR expression, thereby conferring antigen-presenting capacity on the KC.[33] Topical exposure to PK-C agonists such as TPA, aplysiatoxin, and urushiol which produce inflammatory psoriasiform dermatitis,[14] swimmers itch,[34] and poison ivy contact dermatitis[35] respectively, may, via the induction of KC ICAM-1, influence T cell epidermal trafficking and thereby explain the rapid appearance of intraepidermal T cells and cutaneous inflammation. In fact, we have recently observed early KC ICAM-1 expression in poison ivy contact dermatitis as described above.[36]

In summary, our results suggest a new pathophysiological mechanism for inflammatory skin diseases which emphasizes the production of cutaneous immunological events via altered KC signal transduction. Furthermore, the propensity for both cytokine (e.g. IFN-γ), and non-cytokine- (e.g. TPA) mediated mechanisms to induce ICAM-1 on KCs via PK-C-independent and dependent pathways, respectively, may aid the development of new therapeutic agents. Thus, the recently recognized ability of topically applied sphingosine, which is a protein kinase inhibitor, to block TPA-induced inflammation and KC hyperplasia,[14] may be mediated in part through modulation of adhesion molecules and subsequent T-cell trafficking in the skin.

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Cutaneous reactions to substance P and histamine in atopic dermatitis

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SUMMARY

The flare and weal reactions to intradermal injections of histamine and the peptide substance P were measured in a group of patients with atopic dermatitis and compared to reactions in a non-atopic control group. There was no significant difference in the flare areas between the controls and atopics with either reagent. The weal volumes after injection of substance P and histamine were significantly larger in the atopic group. As substance P causes mast cell histamine release, the increased weal volumes produced by substance P in the atopics may be entirely due to the exaggerated atopic weal reaction to histamine.

The undecapeptide substance P (SP) is widely distributed in the central and peripheral nervous tissue where it functions as a neurotransmitter.[1] It has been demonstrated in the upper dermal nerve plexuses where it appears to be localized in the primary cutaneous sensory nerves, and in pig and man, SP-containing nerves have been demonstrated penetrating into the epidermis.[2,3] Antidromic stimulation of afferent cutaneous sensory nerves produces a cutaneous flare and plasma extravasation in the area of skin supplied by the stimulated neurone;[4] pretreatment of the skin with capsaisin which depletes nerve endings of SP prevents this reaction.[5] Intradermal injection of picomolar quantities of SP produces a weal and flare reaction.[6,7] The partial inhibition of this effect by antihistamines and the ability of SP to cause *in vitro* mast-cell degranulation[8,9] suggests that these reactions are at least in part due to SP-induced histamine release. Indeed, intradermal injection of SP in man produces a rise in the level of histamine in the venous blood draining the injected limb.[10]

In spontaneous and suction-induced blisters in a variety of inflammatory dermatoses, SP can be detected in the blister fluid at picomolar concentrations,[11] and at this concentration it is obviously capable of producing weal and flare reactions and itch. At similar concentrations, SP may also participate in inflammatory reactions and may stimulate chemotaxis of human monocytes.[12] It was also recently discovered that SP at 10^{-8} to 10^{-6} M concentrations will stimulate the growth of cultured mouse keratinocytes, and as such may contribute to epidermal proliferation.[13] Patients with atopic dermatitis frequently notice exacerbations of their skin

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inflammation during times of stress, and alterations in the vascular resistance of the skin have been observed.[14] These reactions are similar to those seen with intradermal injections of histamine. To investigate the possible link between cutaneous inflammation and the central nervous system mediated through substance P in patients with atopic dermatitis the weal and flare reactions in a group of patients with atopic dermatitis were compared to the reactions of a group of non-atopic controls.

METHODS

Subjects

Studies were performed on 13 adult patients (mean age 31 years ± 14 years) who have suffered from longstanding atopic dermatitis and who fulfilled the Hanifin–Rajka[15] criteria for this condition. All had avoided taking antihistamines for at least 1 week before the studies began. The 12 control volunteers (mean age 21 ± 5 years) had no personal or first degree relatives with atopic asthma, eczema or allergic rhinitis. Hospital ethical committee approval was granted prior to the commencement of the studies and all volunteers gave written informed consent.

Solutions

All the active agents were made up in a solution of sterile phosphate-buffered saline (PBS) containing 500 mg l^{-1} of bovine serum albumin (BSA) which acted as a carrier protein for the substance P. Plastic laboratory ware and syringes were used throughout to prevent adsorption of histamine.

Powdered SP (Sigma Chemicals Co., Poole, Dorset, U.K.) was diluted in sterile PBS and stored in aliquots at a temperature of −80°C. These were thawed and diluted to the test concentrations 1 hour before injection. Ten, 100 and 200 pmol of SP in 50 μl of diluent and 0·08, 0·16 and 1·6 nmol of histamine acid phosphate (British Pharmacopoeia) in 50 μl of diluent were prepared.

Measurements

Flare area. The maximum diameter of the flare surrounding the injection site, and the diameter of the flare perpendicular to its mid point were measured and the flare area estimated according to the formula in Table 1.

Weal area. The maximum diameter of the weal, and the diameter of the weal perpendicular to its mid point were measured and the weal area estimated according to the formula in Table 1.

Weal thickness. The skin thickness was measured prior to injection (T_0) and at periods after injection (T_1) by pinching and lifting the skin proximal and distal to the injection site so that the jaws of a low tension spring loaded micrometer (Mitatoyo, Japan) could be released onto the weal. Thickness and volume could then be calculated according to the formula in Table 1.

Injections

All subjects were prick tested with the PBS/BSA diluent to determine immediate sensitivity to the BSA, before intradermal injection of 50 μl of the PBS/BSA diluent without active agent. The presence of a weal and flare and changes in the skin thickness at 20 min were recorded. Intradermal injections of the active reagents were introduced into non-eczematous volar forearm skin using a 27 gauge needle.

TABLE 1. Formulae used to calculate weal and flare parameters

Flare area	$\pi/4\,[(D1 + D2)/2]^2$
Weal area	$\pi/4[(d1 + d2)/2]^2$
Weal thickness	$(T1 - T0)/2$
Weal volume	Area × thickness

$D1$ is the maximum flare diameter and $D2$ the flare diameter perpendicular to it. $d1$ is the maximum weal diameter and $d2$ the weal diameter perpendicular to it. $T0$ is the skin thickness prior to intradermal injection of the active agent and $T1$ the skin thickness at the specified time.

Time-response curves

The flare area and weal volume were determined at 1, 3, 5, 10, and 20 min after injection of 100 pmol of SP and 0·16 nmol of histamine.

Dose-response curves

The flare area at 3 min and weal volume at 10 min were calculated after intradermal injection of 10, 100 and 200 pmols of SP and 0·08, 0·16 and 1·6 nmols of histamine.

Statistical methods

Results are expressed as means ± standard error of mean. Flare area and weal volumes in the atopic and the control groups were compared using the unpaired two-tail Student's *t*-test.

RESULTS

Two of the atopic subjects developed a small weal and flare reaction on prick testing with the PBS/BSA diluent and one atopic subject had an increase in skin thickness at 20 min after intradermal diluent injection. These three subjects were excluded and results reported are those of 10 atopic and 12 non-atopic subjects.

Flare area

Intradermal injection of SP produced a flare reaction with a maximal area after 1 min. The flare was faint at first, but as it consolidated and reduced in area, the intensity increased, becoming maximal at 5 min. During consolidation a reticulate border to the flare was commonly seen; measurements of flare diameter were always taken to the most peripheral continuous rim of erythema. Thereafter, the intensity diminished and area regressed as shown in Figure 1.

The 3-min flare area dose-response curve showed a linear trend with increasing flare area with larger doses of SP injection in the atopics, but this clear relationship was not so evident in the control group. The time and dose-response relationships showed no significant difference between the control and atopic groups at any time or dose of SP (Table 2).

Intradermal injection of histamine produced a time- and dose-response curve similar to that seen with SP injection. The mean values for the flare area in the atopics were smaller than that

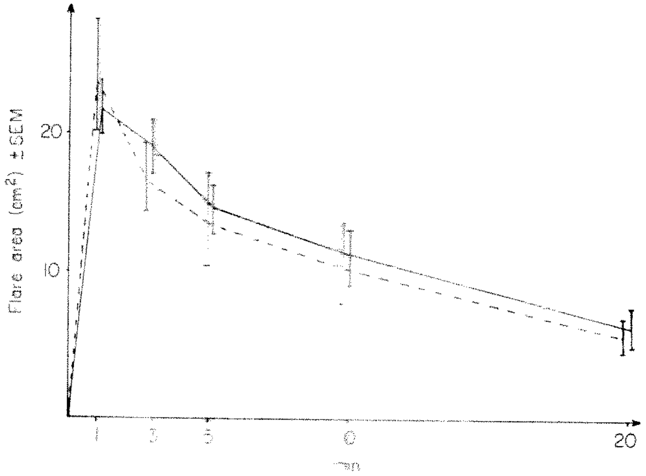

FIGURE 1. Flare area time–response curve for substance P (100 pmol). (——, atopics; - - -, non-atopic controls).

TABLE 2. Three-minute flare area dose–response curve for substance P

	Dose of SP (pmol)		
	10	100	200
Flare area (cm^2) mean ± SEM			
Control	14·9 ± 2·1	17·3 ± 3·0	19·8 ± 3·2
Atopic	9·23 ± 1·5	19·1 ± 1·6	23·7 ± 3·1
	NS	NS	NS

NS, not significant at a level of $P < 0{\cdot}05$.

seen in the controls at 3, 5 and 10 min, but statistical significance at the $P < 0{\cdot}05$ was not reached (Table 3).

Weal volume

Weal volume after SP injection was maximal at 20 min. The time course of the weal reaction in the control and atopics was similar. In both groups there was a linear dose–response relationship in the 10-min weal volume after SP injection (Fig. 2). The weal volumes in the atopic patients were, at the three doses investigated, significantly larger, being double those of the control subjects.

The time course of the weal volumes after histamine injection was similar to that seen after SP injection. Again, in both groups there was a linear dose–response relationship for the 10-min post-histamine injection weal volume (Fig. 3). The weal volumes in the atopic group were significantly larger than the controls at all doses tested.

TABLE 3. Three-minute flare area dose–response curve for histamine

	Dose of histamine (nmol)		
	0·08	0·16	1·6
Flare area (cm^2) mean ± SEM			
Control	10·9 ± 1·2	19·1 ± 2·25	22·4 ± 1·6
Atopic	11·2 ± 2·3	15·3 ± 2·1	21·2 ± 2·2
	NS	NS	NS

NS, not significant at a level of $P < 0·05$.

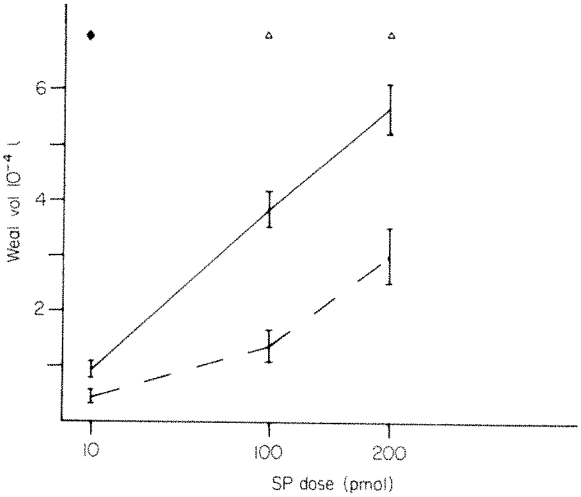

FIGURE 2. Ten-min weal volume dose–response curve after intradermal injection of substance P (——, atopics; – – –, non-atopic controls). △ $P < 0·02$, ◆ $P < 0·05$.

DISCUSSION

The data show that there was no significant difference in the flare area induced by intradermal injection of both SP and histamine between the atopic subjects and the non-atopic controls. In addition the mean flare area for histamine for the atopic group was smaller than the controls at 3, 5, and 10 min, but these differences did not reach significance at a P value of $< 0·05$. Several investigators have commented on the reduced or even absent flare reaction seen in atopic eczema[16–17] after intradermal injection of histamine, though it has been suggested that this occurs only in sites of skin actively inflamed by atopic eczema, and it is not seen in patients with atopic dermatitis when healed[17] or uninvolved[18] sites are studied. Furthermore, when histamine reactions are measured in areas of contact dermatitis diminished flare areas are noted,[18] suggesting that the reduced reaction is secondary to eczematous inflammation, rather than something unique to atopic dermatitis. The choice of uninvolved sites for testing in this study confirms the finding that there is no significant difference between histamine-induced flare reactions in atopics and control subjects.

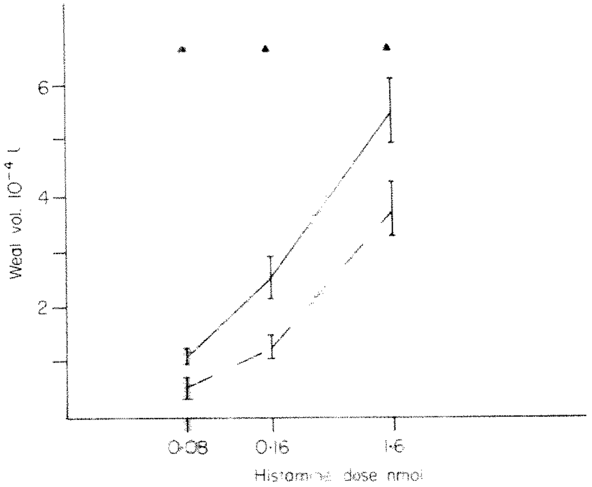

FIGURE 3. Ten-min weal volume dose–response curve after intradermal injection of histamine (——, atopics; – – –, non-atopic controls). ▲ $P < 0{\cdot}05$.

In contrast to the flare reactions, at the three doses of histamine chosen, it was found that the weal volume in the atopics was double that of the controls, and these differences reached statistical significance. Previously weal-volume measurement has only infrequently been performed; Archer *et al.*[19] also noted an increased mean weal volume in the atopics compared to controls with a single dose of histamine, but this did not reach statistical significance. The possibility was considered that the bovine serum albumin, used as a carrier protein in the diluent of the injections, may have had an influence on weal volumes, even in the subjects not developing reaginic reactions to it. In subsequent studies it was found that there was no significant difference in the weal volumes at 10 min produced by 0·1 nmol histamine diluted in PBS alone (mean weal volume $81 \pm 6{\cdot}8$ μl) and 0·1 nmol histamine diluted in PBS and 50 mg l^{-1} BSA (mean weal volume $66 \pm 11{\cdot}2$ μl, $n = 5$, $P = 0{\cdot}5$).

Atopic dermatitis is a condition characterized by severe itch, and the pharmacological mediators responsible for this itch have been extensively studied. There is now considerable evidence that SP mediates its weal and flare reaction by inducing dermal mast-cell degranulation and liberation of histamine. The significantly larger weal response seen after SP at between 10 and 200 pmol doses in the atopics may be entirely the result of the already demonstrated histamine response. In spontaneously arising blisters occurring on eczematous skin, SP has been found at concentrations of the order of 100 pmol. Thus, SP may have a role in the promotion or initiation of the eczematous response in atopic eczema, particularly as it may have twice the capability to produce dermal oedema in atopics as in normal individuals.

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Thermographic assessment of patch-test responses

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SUMMARY

Infra-red thermography was used to quantify, at patch test sites, the allergic responses to experimental preparations of nickel sulphate and primary irritant responses to sodium lauryl sulphate in small groups of volunteers. The technique was also used to assess the patch-test responses in a much larger group of patients who had undergone routine patch testing for contact allergy with a wide range of test substances and among which there were large numbers of allergic, irritant and equivocal reactions. Thermographically, when compared to the surrounding normal skin surface, the sites of allergic reactions appeared as hot areas, the temperature and area of which were apparently dependent on the severity of the response. For allergic responses, there was a good correlation between the clinical assessment and either of two thermographic parameters, temperature and area of involvement. Compared with an aqueous solution of nickel sulphate, 'poor' formulations of the allergen, such as a suspension in soft paraffin base, elicited smaller and cooler reactions. Irritant reaction sites were not 'hot' and the temperature at such sites was no different from that of the surrounding normal skin. Infra-red thermography is a convenient non-invasive technique which apparently can be used to discriminate between irritant and allergic responses and to quantify the latter type of response.

There is continuing interest in methods which can be used to quantify the diverse responses of the skin to various stimuli. As part of an attempt to improve existing patch-testing methodology and to improve the diagnosis of allergic and irritant dermatitis, we have been interested in objective measures of the response of the skin in subjects challenged with topically applied allergens and irritants. There is a need for a simple method to discriminate between allergic and irritant responses especially where these are weak in nature. Many methods, such as laser Doppler flowmetry,[1] photoplethysmography[2] and skin reflectance measurements,[3] have been used to quantify the erythema and hence the severity of the patch-test reactions. More recently, other aspects of allergic and irritant responses have been studied, such as changes in skin-barrier function by means of transepidermal water loss (TEWL) measurements[4] and the associated oedema by ultrasonic methods.[5] At the test site many of these instrumental methods make what is essentially a point measurement so that only a sample of the overall response is provided. Our preliminary studies with infra-red thermography[6] indicated that this technique had the important advantage of providing a 'picture' of the entire response site including the area of involvement and the temperature distribution within it. This paper describes the use of infra-red thermography to quantify the allergic patch-test response and to discriminate between allergic and irritant types.

METHODS

Nickel sulphate, Analar grade ($NiSO_4.7H_2O$, comprising 20·7–21·9% Ni^{2+}, BDH Biochemicals Ltd) was micronized to a mean particle size of < 10 μm before incorporation into the white soft paraffin base by trituration. Sodium lauryl sulphate, SLS (Sigma, Chemical Co., St Louis, MO, U.S.A.), white soft paraffin B.P., WSP (Evans Medical Ltd., Horsham, W. Sussex, U.K.) and hydroxypropylmethyl cellulose, hypromellose (Methocell K4M, Colorcon Ltd., Orpington, Kent, U.K.) were all used as received.

Infra-red thermography

Infra-red (IR) thermographic measurements were made using a Thermovision 720 system having a scanner with an f/1·8, 12° germanium lens and as a reference radiation source, a type 23A reference radiator (both Agema Infra-red Systems U.K. Ltd., Leighton Buzzard, Beds, U.K.). The 720 system is based on refractive silicon optics, in which two rotating prisms provide point by point focusing of the thermal radiation from the skin surface on to a liquid nitrogen cooled indium–antimonide detector. The black and white images on the display screen were recorded photographically with a Polaroid back type 107 camera on professional 667 film. The IR scanner was positioned normal to the patient's back at a distance of 0·7 m. The cross wires of a 1 cm^2 square aperture in a 4 cm^2 perspex frame placed on the patient's back allowed precise focusing of the camera, precise location of the reaction and also a reference linear dimension for the calculation of the area of involvement. Thermography was carried out in a room with a steady ambient temperature of typically 23 ± 1°C.

Evaluation of the thermograms was achieved by using the camera in the isotherm mode, in which isotherms (loci of points having the same temperature) can be superimposed on the infra-red image. Two parameters were recorded at a reaction site, the maximum isotherm level and the area of involvement delineated by the isotherm which marked the separation of the normal uninvolved skin and the 'hot' area. Using the thermal emission of the reference radiator (33 ± 0·1°C) as a temperature standard, the maximum isotherm level was converted to a maximum temperature within the area of involvement. For any particular subject, the response has been expressed as Δt, the difference between the maximum temperature within the area of involvement and that of the surrounding, normal skin surface. The area of involvement, A, was measured by means of a digitizing tablet and interactive pen which was traced around the area on the Polaroid print.

Allergic responses

Nickel sulphate was incorporated into solution vehicle: 1·0, 0·5, 0·2 and 0·1%, in water and 0·2% in 1·0% aqueous SLS and into semi-solid vehicle: 0·2% in 3·0% hypromellose gel and 1·0 and 0·2% in WSP. These preparations were applied to each of 12 volunteer, non-atopic, subjects (10 female, two male; mean age 31·3 ± 15·9 years) who had been diagnosed as nickel allergic on previous attendances at the clinic. The solutions (40 μl) were soaked into filter paper discs in the Finn chambers (Epitest Ltd., Finland) and the semi-solids (12 mg) placed directly in the chambers. The patch-test chambers were applied to the back, under occlusion. The patches were left in position for 48 h and the responses assessed 1 h after removal, both by thermography and visually on the basis of the accepted ICDRG criteria: + + + (3), extreme, vesicular, oedematous and weeping reaction; + + (2), erythematous, palpable reaction; + (1), macular, erythematous reaction; + / − (0·5), equivocal reaction. The values in parentheses were used as clinical scores to subjectively quantify the severity of the reaction at each site and also to calculate a mean clinical response to any particular challenge for the group of 12 subjects.

Irritant responses

Solutions of SLS in water, 5·0, 2·0, 1·0 and 0·5% and a water control were applied using filter-paper discs and Finn chambers to each of 10 normal, volunteer subjects (4 female, 6 male; mean age 28·6 ± 8·6 years) for 48, 24, 12 and 6 h. Two hours after removal of the patches, clinical assessment of the severity of the erythematous response was carried out in a blind manner by two independent observers according to a scale similar to that described by Willis *et al.*[7]: + + + (3), severe erythema; + + (2), moderate erythema; + (1), faint well defined erythema; + / − (0·5), faint ill defined erythema; − (0), no visible reaction. The values in parentheses were used as clinical scores to subjectively quantify the severity of the reaction and to calculate a mean irritant response to any particular challenge for the group of 10 subjects. After the clinical assessment of the test areas, the reaction sites were examined by thermography.

Allergic and irritant responses

In a separate series of observations, the reactions to patch tests on patients attending the clinic for the routine patch-testing procedure for contact allergy were assessed thermographically. The 62 subjects (33 female, 29 male; mean age 39·7 ± 16·1 years) were seen on their second or third visits to the clinic, i.e. 48 or 96 h after application of the patch tests, and had responded to one or more of the test materials. One-hundred and seventy-four reactions, which by the standard clinical criteria comprised allergic and irritant responses of varying degrees of severity, were observed. After the clinical assessment, the severity of the reaction at the response sites was assessed by thermographic measurements carried out by a second, independent observer. The ability of the thermographic parameters Δt or A to allocate a thermographic response to a particular clinical reaction category (allergic or irritant) was tested by the MINITAB® version of linear discriminant analysis.

RESULTS

The clinical and thermographic responses in 12 nickel-allergic subjects to challenge with various nickel sulphate preparations are shown in Table 1. In general terms the results of the clinical evaluation were in agreement with the thermographic assessments.

In the case of the series of nickel sulphate solutions in water, the apparent clinical severity of the response was concentration dependent. Thermographically, all of the allergic responses appeared as hot areas which in any one patient were hotter and larger with higher concentrations of the applied allergen. The thermographic measurements were more sensitive than clinical evaluation. By clinical assessment, discrimination between the responses to different nickel sulphate concentrations in water was not possible. However by thermography, in terms of either Δt or A, significant differences (Mann–Whitney U-test, 95% significance level) between nickel sulphate concentrations could be measured.

TABLE 1. The clinical and thermographic responses in a mixed sex group of 12 nickel allergic subjects (two male and 10 female), after challenge with various preparations of nickel sulphate. Mean responses ± SE of the mean are shown. The clinical responses were meaned by summing the individual ICDRG scores, assigning a value of 0·5 to equivocal reactions, and dividing by the number of subjects (12).

Challenge % $NiSO_4$	Vehicle	Response		
		Clinical	Thermographic	
			Δt(°C)	A(cm^2)
1·0	Water	2·50	1·05 ± 0·11	3·90 ± 1·11
0·5	Water	2·17	0·73 ± 0·13	1·76 ± 0·56
0·2	Water	1·25	0·45 ± 0·11	1·87 ± 0·41
0·1	Water	1·50	0·25 ± 0·09	0·48 ± 0·21
0·2	1·0% SLS	2·50	0·95 ± 0·12	5·39 ± 0·99
1·0	WSP	1·75	0·59 ± 0·09	1·10 ± 0·27
0·2	WSP	0·83	0·00	0·00
0·2	Gel	2·00	0·25 ± 0·11	1·41 ± 0·73

SLS, sodium lauryl sulphate; WSP, white soft paraffin; Gel, 3·0% hypromellose.

Similarly, by thermography, the severity of the elicited response to nickel sulphate could also be shown to be vehicle-dependent and at a concentation of 0·2% nickel sulphate, the vehicles could be ranked SLS > water = hypromellose > WSP (these differences were significant at the 95% confidence level, Mann–Whitney U-test). The allergic response to nickel sulphate (0·2%) in SLS vehicle was most marked and in terms of Δt or A, was the same (Mann–Whitney U-test, 95% significance levels) as that to 1·0% nickel sulphate in water. Clinically, such discrimination was not possible and only the poor performance of the WSP vehicle could be observed. By themselves, WSP, hypromellose and water vehicles produced neither clinical nor thermo-

TABLE 2. The clinical and thermographic responses, in a group of male (M) and female (F) non-atopic, non-nickel allergic subjects, at 48 h after topical challenge with 0·2% nickel sulphate in 1·0% SLS solution and with 1·0% SLS solution (control). Irritant response shown as I.

Patient	Sex	0·2% $NiSO_4$ in 1·0% SLS response		1·0% SLS response	
		Clinical	Thermographic	Clinical	Thermographic
1	M	I	0	I	0
2	F	I	0	I	0
3	M	0	0	0	0
4	F	0	0	0	0
5	M	I	0	I	0
6	F	0	0	0	0

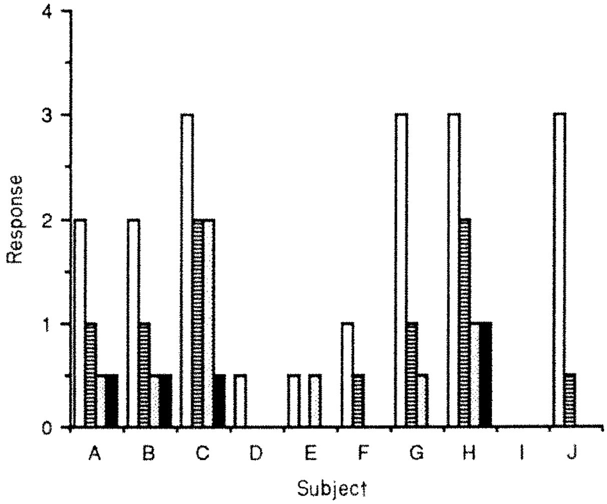

FIGURE 1. The irritant, erythematous, response in a group of 10 normal subjects (six male and four female), to topical challenge for 48 h with various concentrations; □, 5%; ▦, 2%; ▨, 1%; ■, 0·5%, of sodium lauryl sulphate solution.

graphic reactions. SLS produced clinical irritant reactions which were 'cold' thermographically. In non-nickel allergic subjects (Table 2), nickel sulphate in SLS was irritant.

As far as the assessment of the irritant reaction to topical challenge with SLS was concerned, the clinical, erythematous response was found to be concentration dependent (Fig. 1) and in the case of a fixed-challenge concentration of the irritant, 5·0%, was also found to be dependent on the duration of exposure to the irritant (Fig. 2), although in some subjects the longest exposure time of 48 h did not elicit the strongest response. Thermographic assessment of all of these test

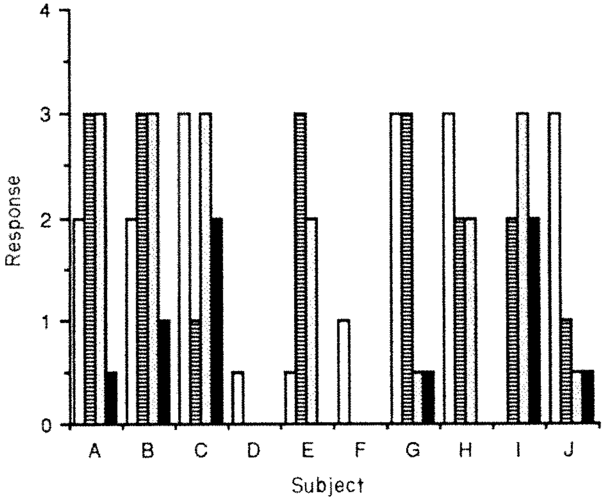

FIGURE 2. The irritant, erythematous, response in a group of ten normal subjects (six male and four female), to topical challenge with 5% sodium lauryl sulphate solution for various contact times; □, 48 h; ▦, 24 h; ▨, 12 h; ■, 6 h.

TABLE 3. The results of clinical and thermographic assessment of the patch-test responses, at 48 or 96 h, in a group of subjects (male and female), attending the CD clinic for routine investigative testing. On the basis of the clinical assessment (ICDRG criteria, allergic, severity 1–3, irritant, I, equivocal, E) the subjects, who had responded to a diverse range of test materials, were allocated to the appropriate clinical response group shown, then assessed thermographically. The mean thermographic responses ± SE of the mean are shown.

Clinical response	n*	Thermographic response: Δt (°C)	Thermographic response: A (cm^2)
3	46	1·00 ± 0·15	3·30 ± 0·32
2	43	0·76 ± 0·07	2·16 ± 0·21
1	29	0·28 ± 0·06	0·65 ± 0·15
E	30	0·22 ± 0·07	0·57 ± 0·19
I	26	0·08 ± 0·05	0·24 ± 0·17

* n, number of responses.

sites, revealed that their temperature was not significantly different from the surrounding, normal, skin surface and that there were no hot areas which were typical of the allergic reaction. Thermographic assessment at several time intervals, 2, 4, 6 and 24 h, after removal of the patches also failed to reveal hot areas. Similarly, in any of the 12 subjects challenged with the various nickel sulphate formulations (Table 1), application of 1·0% SLS vehicle alone as a control did not elicit a thermographic response, although clinically there were five irritant,

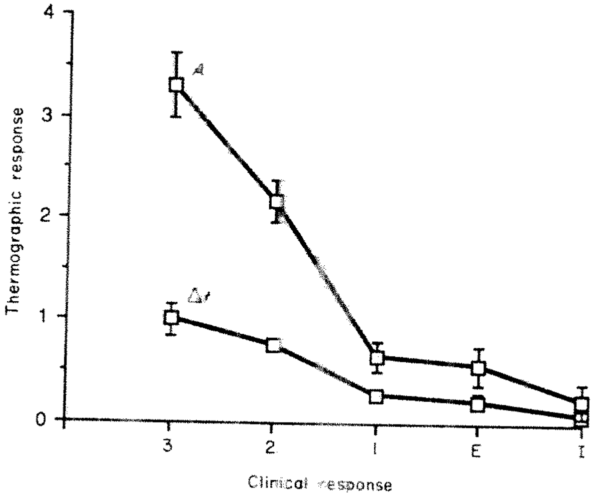

FIGURE 3. A plot of the data in Table 3 to show the relationships between the clinical score and the thermographic assessment of the intensity (Δt, °C) and area of involvement (A, cm^2) of allergic and irritant reactions to diverse topical challenges. The x-axis shows: allergic (severity 3–1), equivocal, E and irritant, I, reactions.

three equivocal and four negative reactions. In addition, in six subjects who were not sensitized to nickel (Table 2), none of the irritant reactions elicited by SLS were 'hot' as determined by thermography. In this latter group, there was no evidence of a synergistic or additive effect between nickel sulphate and SLS in terms of the incidence of the irritant reaction or in terms of the severity of this reaction.

Table 3 and Figure 3 show the results of using the thermographic technique to assess 174 patch-test responses subsequent to routine clinical diagnosis. It can be seen that there was a good correlation between the clinical scoring and the observed temperature and area of involvement, such that the 3+ allergic responses were the 'hottest' and had the greatest area of involvement and those diagnosed as irritant, the 'coldest' with the smallest area of involvement. Δt and A were equally effective in differentiating between allergic and irritant reactions. Linear discriminant analysis showed that either of the thermographic parameters, Δt or A, could be used to classify a response as allergic or irritant and in this respect the extent of the agreement with the clinical diagnoses was 80% for allergic and 92% for irritant reactions.

DISCUSSION

It is generally accepted that the severity of the patch-test response in subjects tested with known allergens is proportional to the concentration of allergen applied, so that the severity of the responses to different formulations of nickel is a measure of the topical bio-availability of the allergen from these formulations. Thus, WSP was a 'poor' vehicle for nickel sulphate since the salt is present as an insoluble solid and thermographically, 1·0% nickel sulphate in WSP elicited as severe a response as 0·2% nickel sulphate solution. In the hypromellose gel however, the nickel sulphate is present in solution form and in this vehicle elicited a more severe reaction. There was a greater topical bio-availability than with the same concentration of 0·2% allergen in WSP. The marked allergic response to 0·2% nickel sulphate in 1·0% SLS vehicle was a novel finding and presumably is an indication, at least in part, of the ability of this surfactant to enhance penetration of the allergen to the effector cells of the immune response. Enhanced penetration is in accord with the marked ability of SLS to disrupt skin barrier function as measured by TEWL.[8] In this regard, the large area of involvement associated with the SLS vehicle, which was revealed by thermography, is significant and probably represents an increased lateral spread or penetration of the allergen. Further support for a purely penetration-enhancing role for SLS comes from the observation that in nickel allergic subjects, the irritant effects of 1·0% SLS on its own were as expected for this concentration and in subjects with no nickel allergy, nickel sulphate had no effect on the irritant reaction to topical SLS. In nickel-sensitive subjects however, it may be that the apparent high potency of the combination of nickel sulphate in SLS also represents the result of an *in situ* interaction between the irritant and allergic responses.

Although thermography provided more information than clinical assessment and could be used to quantify the responses to topical nickel challenge in allergic subjects, its ability, on the basis of the present data, to discriminate between 1+ allergic and equivocal reactions, was apparently poor. The equivocal reactions present the greatest problem in diagnosis. This lack of discriminatory power was in part due to the apparent variability of the thermographic data and so for example, the differences between the mean thermographic values, of Δt and A, for allergic responses scored clinically between 3+ and 2+, were not statistically significant.

It must be stressed, however, that the data in Table 3 and Figure 3 represent comparisons of the subjective, clinical scorings with the objective thermographic measurements. In effect, responses were assigned to the various clinical score categories; allergic (3, 2, or 1), irritant or equivocal, prior to the thermographic measurements and it is instructive to examine this procedure as a source of the apparent variability of the thermographic data. There was of course inter-subject variation in the thermographic response which was perhaps compounded by the fact that the thermographic measurements were carried out on test sites at 48 or 96 h after application of the patches and that a wide diversity of allergens was involved. However, probably the most important contributory factor to variability in the thermographic data was clinical misdiagnoses. Thus, the group of responses scored clinically as allergic 3 +, included two which were not 'hot' by thermography and which might therefore on the basis of thermography be described as irritant rather than allergic reactions. Similarly, the erroneous allocation of a weak allergic response to the clinically irritant group would greatly increase the variability of the thermographic scoring of the latter type of response.

The extensive areas of involvement, characteristic of severe allergic reactions, which could only be observed by thermography, were large enough to encompass adjacent patch-test sites if the standard spacing of the Finn chambers was used. This observation may be of clinical significance in that during routine use, with the standard spacing between patches, a severe response may as a consequence prejudice the nature of the responses at adjacent sites.

The most interesting finding was the apparent ability of thermography to differentiate between allergic and irritant responses, the former being 'hot' and the latter 'cold'. Although the controlled studies have involved only one allergen, nickel sulphate, and one irritant, SLS, the thermographic observations of the responses to diverse allergens and irritants provide confirmation of the findings made with these two test agents. It is also significant that a measurable thermal response to SLS could not be obtained over a range of SLS concentrations, a range of contact times with the irritant and for either of these conditions, an assessment at various times after removal of the patches. Although the reason for the thermographic difference between allergic and irritant responses is not immediately apparent it presumably reflects underlying differences at the cellular or subcellular level. Several studies[9–14] have failed to identify any major qualitative differences between these reactions in terms of the types of cell appearing or being recruited to the site or in terms of the apposition of interacting cells such as lymphocytes and Langerhans cells. There is some evidence however that the number of Langerhans cells at the reaction site is decreased in an irritant response and in an allergic response is initially increased before reverting back to normal.[15,16]

Some of the described differences between allergic and irritant reactions may, however, have a bearing on the thermographic observations described here. Allergic reactions are associated with an increased production in the skin of arachidonic acid and some of its metabolites such as prostaglandin E_2 and in some subjects leukotriene B_4, which is not the case in irritant reactions.[17] The extent of perivascular dermal infiltrate is much smaller at irritant response sites than at allergic response sites[16] and high-frequency ultrasound measurements[5] show that the extent of the oedema of allergic responses is greater than the smaller, more superficial oedema of an irritant response. TEWL has been described as a means of differentiating between allergic responses to nickel in sensitive individuals and irritant responses to SLS[4] and a characteristic of the latter is an increased TEWL, which was not observed for allergic responses except for severe reactions caused by prolonged exposure to the allergen when the decreased barrier function observed was probably a secondary manifestation of the inflammatory response.

Thermography provides information on the radiative heat loss from the skin surface.[18] An area such as an allergic reaction site which appears 'hot' is at a higher equilibrium temperature than surrounding skin because of increased heat transport from the core as a result of local vasodilation and/or increased local metabolic activity. On this basis it was to be expected that a high temperature would be found at the site of an allergic response and indeed allergic response sites which were 'hot' by thermography have been described.[19,20] The lack of a thermal response at the irritant response site was not expected and this finding is at variance with that of Brånemark *et al.*[19] who reported that irritant responses were 'hot'.

It is not immediately apparent why we were not able to detect an irritant response by thermography. However we have observed that at a severe allergic response site where the skin is broken, any drops of tissue exudate appear, by thermography, as 'cold' spots within the hot area of involvement. Similarly small drops of water placed on the surface of normal skin appear 'cold' by thermography, presumably as a result of evaporative cooling. It may be then that the increased TEWL associated with an irritant reaction[4] provides sufficient evaporative cooling to counteract any heat production arising as a result of metabolic activity or vasodilation, allowing the response site to maintain a neutral heat balance so that its temperature is not detectably different from that of the surrounding normal skin.

Infra-red thermography is a sensitive, non-invasive technique which provides a means of quantifying allergic reactions and the interaction between the allergen and the vehicle used to apply it to the skin of the allergic subject. It would seem that it also provides a means of discriminating between allergic and irritant reactions and evaluating equivocal responses.

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The influence of microwave radiation on transdermal delivery systems

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SUMMARY

It has been alleged that the exposure of a transdermal delivery system to leakage of microwave radiation from a domestic microwave oven can result in the user receiving a second-degree burn in the area of the patch. Several transdermal delivery systems were exposed to microwave radiation from an Electro Medical Supplies Microtron 200 microwave diathermy unit. Temperature rises of up to 2·2°C were recorded at a maximum power density of 800 W/m^2. These temperature rises were considered insignificant compared to that required to produce a burn. The exposure of transdermal delivery systems to a microwave diathermy field or lower level leakage radiation from a microwave oven is unlikely to cause direct thermal injury to the wearer.

Systems for the sustained, long-term transcutaneous delivery of drugs are becoming increasingly popular in the treatment of a variety of disorders. One such system is Transiderm Nitro-10 (Ciba-Geigy Pharmaceuticals, Horsham, W. Sussex, U.K.), a preparation of glyceryl trinitrate contained in a patch which is applied directly to the skin for prophylaxis of angina pectoris. The average absorption rate is 10 mg per 24 h. The potential hazard associated with glyceryl trinitrate and defibrillation has been recognized[1,2] and the data sheet accompanying the product carries a precautionary statement in this regard.

A case has been reported in which a 51-year-old man presented with a second-degree burn in the area of his Transiderm Nitro-10 patch.[3] The patient had been sitting next to a domestic microwave oven when it was switched on and it is alleged that the burn was caused through heating of the adhesive strip of aluminized plastic in the patch by a leakage of microwave radiation. Unfortunately, the power density of the stray field was unknown, but if transdermal delivery systems can couple into leakage fields from domestic microwave ovens and cause burns, then this would constitute a serious drawback to their usefulness. Also, exposure to the microwave diathermy fields as used in physiotherapy, which are many times greater in magnitude, would be a cause for concern.[4]

The purpose of this investigation was to determine the temperature rises induced in transcutaneous delivery patches, including Transiderm Nitro-10, exposed to conditions of known field strength from microwave diathermy equipment similar to that used in physiotherapy departments.

METHODS

The microwave source was an Electro Medical Supplies Microtron 200 microwave diathermy unit emitting radiation at 2·45 GHz. Power density was mapped using a Narda Isotropic radiation monitor, comprising a model 8633 probe and model 8611 metering instrument. This is sensitive within the frequency range 10 MHz–26 GHz with a dynamic range of 1·0–1000 W/m^2.

Four commonly used patches were irradiated: Transiderm Nitro-10 (Ciba Geigy); Scopoderm (Ciba-Geigy); Estraderm (Ciba-Geigy); and Deponit (Schwarz Pharma, Chesham, Bucks, U.K.).

The experiment was conducted in a chamber lined with Eccosorb loaded dielectric material in order to minimize reflections. The power density of the microwave field was mapped and then each patch was placed at a distance of 0·1 m from the applicator at a position of maximal field strength. The patches were exposed for 5 min to a range of powers up to the maximum available (200 W) corresponding to a power density range of 140–800 W m^2. The temperature was measured before and immediately after exposure using a thermocouple probe, which was removed while the field was applied.

RESULTS

The results are shown in Figure 1. Temperature rise increased with power density. The four patches registered temperature rises of 1·5–2·2°C at the maximum power density of 800 W/m^2.

DISCUSSION

Three of the four patches investigated have adhesive strips of aluminized plastic and one (Estraderm) has no metallic element. The heating of the patches may have been caused, at least in part, by oscillations of polar molecules in the drug preparation under the influence of the

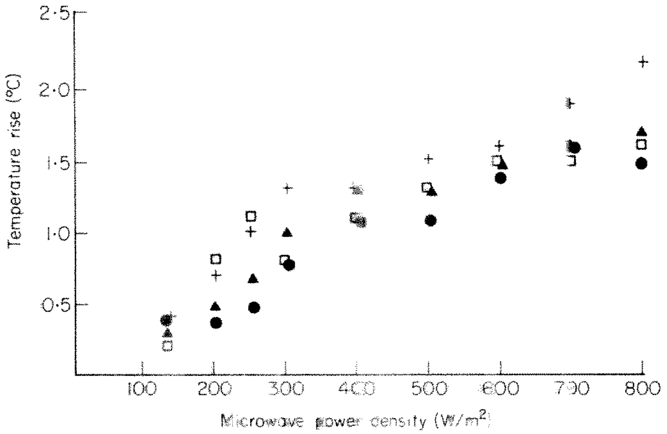

FIGURE 1. Temperature rise of the transdermal patches against power density of the microwave field. □, Deponit; ▲, Scopoderm; ●, Transiderm Nitro; +, Estraderm.

electric component of the microwave field. This would occur without any metallic element being present. All four samples, including Estraderm, showed a clear relation between temperature rise and power density.

It was alleged that the aluminized plastic of a Transiderm Nitro-10 patch coupled into the microwave field leaking from a domestic microwave oven and this induced a temperature rise sufficient to cause a second-degree burn, but our results do not support this. The British Standard 5175[5] sets a maximum permitted level of 50 W/m^2 for the power density at a distance of 50 mm from a microwave oven. We have recently reported results from a total of 662 surveys of microwave ovens over an 8-year period[6] in which the maximum power density recorded at a distance of 50 mm from the oven operating with the door closed was 200 W/m^2. According to the results of the present study, this would cause a temperature rise of less than 1°C in the patch. The highest level of leakage radiation recorded during the reported survey while opening the door of an oven was 600 W/m^2 at 50 mm and which would induce a temperature rise of only 2°C in a transdermal patch.

Of greater concern is the possible danger of exposure to microwave diathermy in physiotherapy departments. Our results show that at the maximum power setting of 200 W, the temperature rise was less than 3°C. This rise is insignificant compared to that which would produce a burn.

We conclude that the temperature rise induced in Transiderm Nitro-10, Scopoderm, Estraderm and Deponit by leakage from a microwave oven or under direct exposure during microwave diathermy would be unlikely to cause direct thermal injury to the wearer.

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Uroporphyrinogen decarboxylase deficiency in hepatoerythropoietic porphyria: further evidence for genetic heterogeneity

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SUMMARY

Catalytic and immunoreactive erythrocyte uroporphyrinogen decarboxylase was measured in a woman with hepatoerythropoietic porphyria (HEP). The uroporphyrinogen decarboxylase activity was 24% of the mean value for normal controls and the concentration of the immunoreactive enzyme (106 ng/mgHb), measured with the rocket immunoelectrophoresis technique, did not differ from that of healthy controls. Consequently, catalytically inactive, cross-reactive immunological material (CRIM) was present, and the patient was CRIM-positive. This enzyme activity and immunoreactive enzyme concentration differs from those for previously known HEP patients, and represents a new mutation, evidence for heterogeneity in inherited uroporphyrinogen decarboxylase deficiency.

Hepatoerythropoietic porphyria (HEP) is a rare variant of porphyria cutanea tarda (PCT) and to date, a total of 16 cases have been reported.[1–14] On the basis of family examinations,[15] it was shown that HEP was probably the homozygous form of familial PCT, and this was later confirmed.[10,11,14] The characteristic enzyme deficiency is the same in both PCT[16] and HEP[15]: a decrease in the activity of uroporphyrinogen decarboxylase. Whereas the enzyme activity in familial PCT is about 50% of the normal level,[10,15,17] in HEP the decrease is more marked.[10–12,14,15] The catalytic activity and the immunologically measured enzyme protein concentration revealed the existence of two different groups. In one group of HEP patients, the immunoreactive protein was decreased to the same extent as the catalytic activity (cross-reactive immunological material (CRIM)-negative mutation).[10,18] In the other HEP group,[19] the reduction in catalytic activity was not accompanied by a parallel decrease in the concentration of immunoreactive enzyme. Catalytically inactive CRIM was present (CRIM-positive mutation). Recently, a uroporphyrinogen decarboxylase mutation was reported[20] in which the enzyme

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protein concentration was diminished to a level lower than 7% of that in healthy controls, while the enzyme activity was higher than expected at 16%.

An investigation is reported of the catalytic activity of uroporphyrinogen decarboxylase and the level of immunoreactive enzyme protein in a HEP patient in whom the enzyme deficiency proved to be a mutation which differed from all previously known cases.

METHODS

Patient

The patient is the female of siblings with HEP who were first presented as unusual cases of familial porphyria and have previously been reported.[6]

Methods

Urinary and faecal porphyrins were analysed by means of thin-layer chromatography;[21,22] erythrocyte porphyrins were determined fluorimetrically.[23,24]

Uroporphyrinogen decarboxylase activities were measured in haemolysates of anti-coagulated whole blood samples, with pentacarboxyl-porphyrinogen III as substrate. Immunoreactive uroporphyrinogen decarboxylase in the erythrocytes was determined by electroimmunoassay in agarose gels (rocket immunoelectrophoresis) with a rabbit antiserum to human erythrocyte uroporphyrinogen decarboxylase. The rockets were visualized by autoradiography. Uroporphyrinogen decarboxylase concentration was determined with purified human erythrocyte enzyme as the standard.[25]

RESULTS

Sclerodermoid skin changes have been the most characteristic clinical feature of our patient over the 20 years of follow-up.[6] These changes have slowly worsened with time. Although the porphyrin excretion of the patient has changed slightly, the porphyrin pattern characteristic of HEP has been maintained throughout the whole period.

The average urinary total porphyrin level was found to be 5579 μg/day; over 20 years, values above 10 000 μg/day have been measured several times. The dominant fractions were uro- and

TABLE 1. Porphyrin analyses

Urinary (μg/day)	4-COOH-porphyrin (copro + isocopro)	306
	5-COOH-porphyrin	209
	6-COOH-porphyrin	379
	7-COOH-porphyrin	2398
	8-COOH-porphyrin	2287
Total (μg/day)		5579
Faecal total porphyrin (μg/g) (dry sample)		81
Plasma porphyrin (μg/dl) (uro)		29
Erythrocyte porphyrin (μg/dl) (42% free and 58% zinc-proto)		466

TABLE 2. Erythrocyte uroporphyrinogen decarboxylase activity with pentacarboxyl-porphyrinogen III as substrate

Healthy controls ($n = 20$)	14·9–30·2 pmol/min per mgHb
Average	19·7
Our HEP patient	4·8 pmol/min per mgHb

heptacarboxyl-porphyrin, but the levels of other fractions were also markedly elevated. As in the faeces, the 4-carboxyl-porphyrin was a mixture of coproporphyrin and isocoproporphyrin. The faecal total porphyrin level was only slightly enhanced. Porphyrin levels in the blood were strongly elevated, with uroporphyrin in the plasma, and protoporphyrin in the erythrocytes. The erythrocyte porphyrin was present as free and zinc-chelated protoporphyrin. The results of porphyrin analyses are summarized in Table 1.

As expected from the dominant fractions (uro- and heptacarboxyl-porphyrin), the uroporphyrinogen decarboxylase activity was 4·8 pmol/min per mg haemoglobin, which was significantly lower than that in healthy controls (Table 2). The immunoreactive uroporphyrinogen decarboxylase level in the haemolysate of the patient was 106 ng/mg haemoglobin, which was almost the same as the mean value for healthy controls (Fig. 1). In our HEP patient, enzyme

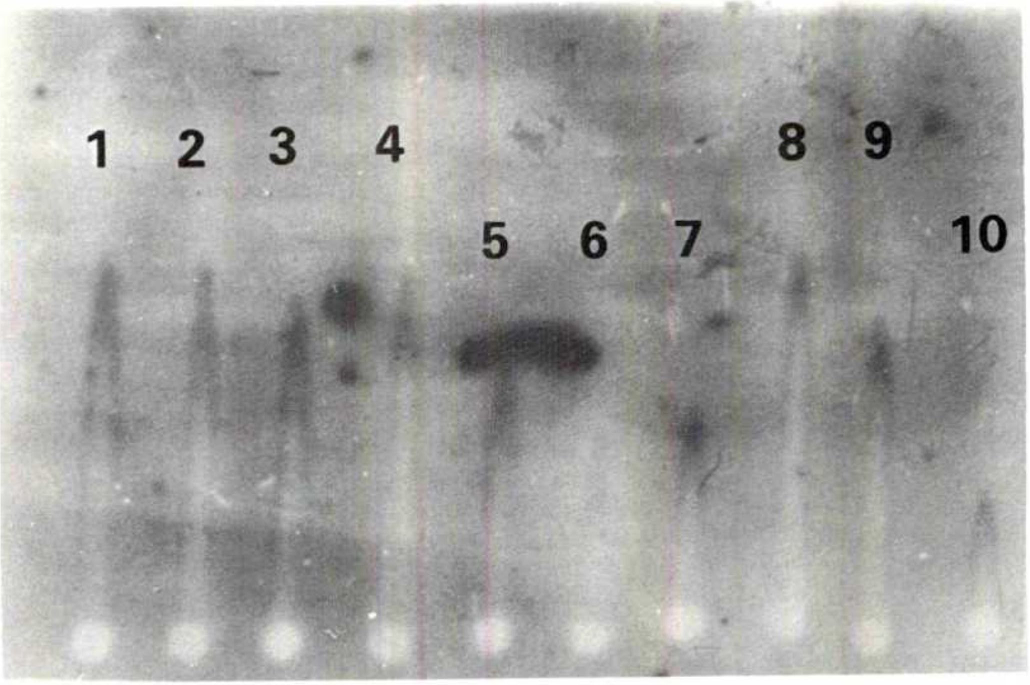

FIGURE 1. Rocket immunoelectrophoresis of erythrocyte uroporphyrinogen decarboxylase. 1, 3, 5, 7, 10 = standards containing 86, 69, 53, 35 and 18 ng enzyme; 2, 4, 8 = parallel samples from patient with HEP; 6 = familial PCT (67 ng/mg Hb); 9 = healthy control (121 ng/mg Hb).

protein was present in a similar concentration to the controls, but its catalytic activity was markedly lower than normal. The patient is CRIM-positive (Table 3).

DISCUSSION

To date, uroporphyrinogen decarboxylase activities in patients with HEP have been measured by several authors (Table 4). In the majority of HEP patients investigated so far, the uroporphyrinogen decarboxylase activity ranged between 3 and 13% of that in controls (average 7%). A considerably different enzyme activity has been reported previously only by Toback *et al.*,[14] in a single case, in whom the activity was 27% of the normal value (or 16%, based on the initial velocity of the enzyme reaction).[14,20] The erythrocyte uroporphyrinogen decarboxylase activity in our patient was 24%, which was similar to that in the case of Toback *et al.*,[14] and markedly differed from the values in other HEP patients. This 24% activity is relatively high for HEP, but differs from the values for familial PCT cases where there is usually a 50% decline in activity.[15]

Immunological investigations of uroporphyrinogen decarboxylase in patients with familial PCT have so far proved these cases to be CRIM-negative.[10,17] As shown in Table 4, some HEP patients were also CRIM-negative. In the CRIM-negative mutation, the catalytic activity of the enzyme and the quantity of immunoreactive enzyme protein are decreased to the same extent (average 7% of normal value), and the activity/mass ratio was 1. In our patient, the immunoreactive enzyme concentration was that found in healthy controls, and only the catalytic activity of the enzyme was lowered. The activity/mass ratio was < 1 (CRIM-positive mutation). CRIM-positivity has previously been found in the HEP patients reported by Lim and Poh-Fitzpatrick,[12] and investigated for this purpose by Sassa *et al.*[19] In these cases the enzyme activity was decreased by approximately the same extent (to 13·6 and 8·8%) as is usual in other HEP patients (see Table 4) and our patient, with her higher enzyme activity, was clearly different from them. The results for our patient also differed from those of the 'mild HEP patient' investigated by Toback *et al.*[14] and by Fujita *et al.*,[20] where in both the proband and the proband's parents and descendants, the enzyme activity was decreased to a lesser extent than the immunoreactive protein, and where the activity/mass ratio was > 1. Our patient, with a 24% enzyme activity and CRIM-positivity, differed from all the HEP patients so far tested, and represents a new mutation for the deficiency of uroporphyrinogen decarboxylase.

The characteristic clinical features of our patient were marked sclerodermoid changes with mutilation, hypertrichosis and extremely elevated porphyrin excretion,[6] as well as increased levels of free and zinc-chelated protoporphyrin in the erythrocytes and only a slight response to phlebotomy therapy. The patient had 24% activity of uroporphyrinogen decarboxylase and was CRIM-positive.

TABLE 3. Immunoreactive uroporphyrinogen decarboxylase concentrations in the erythrocytes (ng/mg Hb)

Controls (*n* = 20)	77–146	
Average	107	
Our HEP patient	106	CRIM-positive

TABLE 4. Abnormalities in the catalytic and in the immunoreactive enzyme protein concentration of uroporphyrinogen decarboxylase in HEP patients

				Erythrocyte uroporphyrinogen decarboxylase	
Case	Author	Age (year)	Sex	Activity (% of normal control)	Activity/mass ratio (ratio to normal control)
1	Gunther[1]	16	F	—	—
2	Aguade *et al.*[2]	42	F	6·9	—
3	Hofstad *et al.*[3]	4	M	—	—
4	Brenan;[2] Czarnecki[8]	3	F	8*	—
5	Aguade *et al.*[5]	8	F	6·9	—
6	Simon *et al.*[6]	60	M	—	—
Currently examined case:					
7	Simon *et al.*[6]	57	F	24	< 1 (CRIM-positive)
8	Hönigsmann and Reichel[7]	63	F	—	—
9	Day and Strauss[9]	12	M	—	—
10	de Verneuil *et al.*;[10] Sfar *et al.*[18]	7	F	5·2	= 1 (CRIM-negative)
11	de Verneuil *et al.*;[10] Sfar *et al.*[18]	7	F	5·2	= 1 CRIM-negative)
12	Lazaro *et al.*[11]	2	F	2·8–3·2	—
13	Lim and Poh-Fitzpatrick;[12] Sassa *et al.*[19]	7	M	13·6	< 1 (CRIM-positive)
14	Lim and Poh-Fitzpatrick;[12] Sassa *et al.*[19]	9	M	8·8	< 1 (CRIM-positive)
15	Bundino *et al.*[13]	10	M	7·3	—
16	Toback *et al.*;[14] Fujita *et al.*[20]	31	M	27–28†	> 1

* Measured in fibroblasts.
† Or 16% based on the initial velocity.

It has been suggested[14] that HEP patients with a mild uroporphyrinogen decarboxylase deficiency are not homozygous for a single defect at the uroporphyrinogen decarboxylase gene locus, but are double heterozygotes. This may be the case in our patient, but there are no other family members available for investigation. However, this case is further evidence of heterogeneity in uroporphyrinogen decarboxylase deficiency.

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Factor XIII in scleroderma: *in vitro* studies

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SUMMARY

The administration of Factor XIII (FXIII) produces a beneficial effect on the skin lesions in about 50% of the treated patients with progressive systemic sclerosis (PSS). The effect of FXIII on various skin fibroblast functions (proliferation, attachment, biosynthetic activity and mechanical properties) was investigated *in vitro* using normal and PSS strains. In cell culture, most of the PSS fibroblast strains synthesized excessive amounts of collagen. Other cell functions such as adhesion to collagen I or III, to fibronectin, retraction of collagen lattices, proliferation in low serum concentration and degradation of newly synthesized collagen were not significantly different. The addition of FXIII (1 U/ml) inhibited the synthesis of collagen by normal fibroblasts and reduced it in PSS fibroblasts to a level similar to that of normal fibroblasts. This effect was observed for cells cultured on plastic or in a collagen lattice. In the latter, an increased amount of collagen degradation was observed. No significant effect of FXIII on the other cell functions was noted. Excessive collagen production by PSS fibroblasts can be repressed by FXIII *in vitro* by at least two distinct mechanisms: a reduction of collagen synthesis and an increased degradation of the newly synthesized collagen.

Scleroderma (progressive systemic sclerosis (PSS)) is a clinically variable disease characterized by excessive accumulation of collagen in various tissues leading to sclerosis of the skin, internal organs and blood vessels.[1,2] The aetiology of such collagen accumulation is not known. Some authors found no difference in collagen synthesis between normal and scleroderma fibroblasts *in vitro*[3] while others found substantial evidence that collagen synthesis by scleroderma fibroblasts is abnormally elevated.[4] This observation suggests either a defect in the regulation of collagen synthesis by scleroderma fibroblasts or a selection of high collagen producing cells from heterogeneous population of fibroblasts in the scleroderma dermis.[5] More recent work suggests a microvascular origin in the pathogenesis of PSS.[6]

Although many different therapies have been tried in the treatment of scleroderma, all have failed to completely cure the patients. Factor XIII (FXIII) was first used in 1975,[7] and the results of a long-term follow-up of 86 PSS patients treated by intravenous administration of

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FXIII have been recently published.[8] A lasting effect was observed in one half of the treated patients with improvement of the skin but not of visceral lesions.

FXIII is the transglutaminase of blood coagulation and is able to covalently cross-link several fibrous proteins such as fibrin to fibrin,[9] fibronectin to fibronectin,[10] fibronectin to fibrin,[11] fibronectin to collagen,[12] collagen to collagen[13] or macromolecules to other proteins.[14–18] *In vitro*, FXIII has been found to modulate several cell functions. It enhances the fibronectin-mediated attachment of transformed pulmonary embryonic rat epithelial cells to collagen I[19] and allows them to retract a three-dimensional collagen lattice.[20] FXIII stimulates the fibronectin-mediated attachment of fibroblasts to fibrin and their proliferation on this substrate.[11] It has also been reported to enhance the multiplication of mesenchymal cells[21] of vessel wall and tumour cells.[22] FXIII also inhibits the migration of epidermal cells on a plastic support[23] and makes fibrin chemotactic for skin fibroblasts (personal observation). Our recent data demonstrated that FXIII stimulated collagen synthesis by fibroblasts included into three-dimensional fibrin gels but inhibited collagen synthesis by fibroblasts cultured on plastic or in a collagen lattice.[24]

This study compared various functions of normal and PSS fibroblasts, with and without exposure to FXIII, *in vitro* and *in vivo*.

METHODS

Materials

The following reagents were used: methyl-[^{3}H]-thymidine (3·4 Ci/mmol) and 5-[^{3}H]-proline (10 Ci/mmole) (New England Nuclear, Boston, MA, U.S.A.); culture media and trypsin solution (Gibco, Grand Island, NY, U.S.A.); and fetal calf serum (Gibco, and Flow Laboratories, Brussels, Belgium); tissue culture plates, 24 well-plates for attachment assay and bacteriological dishes (NUNC); factor XIII subunit A (62·5 U/ml) and thrombin (30 U/ml) (Behringwerke) and bis-benzimidazol H33258 (Hoechst). Plasma fibronectin was purified using the method of Yamada[25] and was more than 95% pure. Collagen types I and III were purified from fetal bovine skin as previously described[26] and kept sterile at a concentration of 1 mg/ml in 0·1% acetic acid at −20°C. For the preparation for cell attachment support the collagen was previously dialyzed against 0·01 mol/l NaH_2PO_4/Na_2HPO_4, 0·3 mol/l NaCl, pH7·4. FXIII was activated (FXIIIa) by adding 0·1 U of thrombin per U of FXIII in serum-free Dulbecco's modified Eagle's Medium (DMEM) with incubation for 15 min before addition to the culture medium.

Cell culture

Skin fibroblast cultures were initiated by cell outgrowth from explants dissected from biopsies taken from the forearm (F) or the abdomen (A) of healthy volunteers or from patients with scleroderma (PSS). When indicated, reticular (R) and papillary (P) dermis were separated by dissecting the skin before using the explants. Informed consent was obtained from all the patients.

The cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS), ascorbic acid (50 μg/ml), penicillin–streptomycin (100 U/ml) and fungizone (2·5 μg/ml) and transferred at 1/3 dilution after trypsinization (0·1% trypsin, 0·02% EDTA) when reaching confluence. Twenty-four hours before use, the medium was replaced by bicarbonate-buffered DMEM supplemented with 10% FCS, glutamine, ascorbic acid, penicillin and streptomycin as above. In all experiments, fibroblasts were used at a low population doubling level between passages 3 and 8.

Preparation of collagen lattice

Collagen lattices were prepared in the absence and presence of FXIIIa as previously described.[20]

Cell attachment assay

Preparation of coated collagen I and III polymers and fibronectin as attachment supports as well as the attachment assay were performed as previously described.[19]

DNA measurements

The DNA content was measured by the fluorimetric technique of Labarca and Paigen[27] on aliquots of sonicated cells or lattices.

Cell proliferation measurements

The effect of FXIII on multiplication of normal and PSS fibroblasts was tested on sparse (2000 cells per cm^2) and on 2-day post-confluent cultures. Cells were made inert by culturing them from 18 h in DMEM supplemented with 0·1% FCS (low serum medium) and, when indicated, 1 U/ml of FXIIIa was added. Labelling was then performed for 8 h by adding [^{3}H]-thymidine (3·75 μCi/ml) in fresh low serum medium. After washing and sonication of the cells, DNA content was determined as above and thymidine incorporation was measured by precipitation with cold trichloroacetic acid (TCA, 30% final concentration) 1 h at 4°C. The precipitate was collected on Whatman GFA filters and the retained radioactivity measured in a LKB liquid scintillation spectrometer.

Metabolic labelling

Protein and collagen synthesis were determined by isotopic labelling with [^{3}H]-proline (10 μCi/ml) as previously described for monolayers of cells or collagen lattices.[24] Labelling for cells in the monolayer was performed 2 days after confluence and for cells in lattices, 1 day after forming the lattice.

Patients, treatment and clinical studies

Three patients with generalized scleroderma were selected for treatment with FXIII. All three consented to a skin biopsy before and after two courses of therapy with FXIII.

Patient 1. A 41-year-old road worker had suffered from Raynaud's phenomenon for 2 years. His hands were swollen and cyanotic and the mobility of the joints was limited and he had multiple ulcers of the pulps of the digits that had left pitted scars. He had moderate dyspnea and a restrictive and obstructive lung disease. The lower third of the oesophagus showed slow peristalsis and the chest X-rays showed interstitial fibrosis of both lungs. X-rays of the hand revealed distal erosions of the second and third digits. Capillaroscopy was compatible with scleroderma and the skin biopsy of the forearm was suggestive of the disease. Speckled antinuclear antibodies were present in moderate titre. After receiving 1250 U of FXIII daily (Fibrogamin®, Behringwerke) by i.v. for 15 days the suppleness of the skin had increased and his cyanosis was less marked. The second course of FXIII (1250 U/day for 16 days) was administrated 1 month later. Following this treatment the improvement of his skin persisted and the motility of the oesophagus improved though the lung function studies showed no change.

Patient 7. A 19-year-old Tunisian girl presented with sclerosis of the extremities and Raynaud's phenomenon that she had suffered from for 6 years. Over the last year the sclerosis had progressed to involve almost all the skin. The mobility of the tongue was reduced. She also had restrictive lung disease with reduced diffusion and erosions on the distal parts of all her digits, a marked reduction in the motility of the lower two-thirds of the oesophagus and extensive sclerosis of the dermis. Speckled antinuclear antibodies were positive. The skin biopsies showed extensive sclerosis of the reticular dermis and the hypodermis. After receiving the two courses of FXIII, 1250 U/day for 21 days and 15 days, most of the abnormal signs were unchanged apart from an improved motility of the oesophagus.

Patient 8. A 22-year-old girl presented with dyspnea and had suffered from Raynaud's phenomenon of both hands for 1 year. She had undergone cervical sympathectomy on both sides. She was asthenic, had lost 15 kg and complained of stiffness of the extremities and had dysphagia. A skin biopsy showed advanced scleroderma. The motility of the lower oesophagus was reduced and lung function tests indicated restriction with reduced diffusion. Chest X-rays showed an interstitial fibrosis. The bones of the fingers were osteoporotic at their ends. Speckled type antinuclear antibodies were present. The capillaries of the nail-bed were reduced and ectatic. After receiving FXIII 1250 U/day for 15 days and a second course of 16 injections of 1250 U 1 month later she had a subjective improvement of the stiffness of the skin and was less weak, but all the other signs were unchanged.

Patients 9, 10, and 11. These had systemic scleroderma and were observed by T.Krieg *et al.*[28] The cultures of fibroblasts were taken from skin of involved areas.

RESULTS

The percentage of adhesion (Fig. 1) on type I and type III collagen and on fibronectin did not significantly differ between normal and PSS fibroblasts. FXIIIa did not modify attachment. When included into collagen lattices, normal and PSS fibroblasts retracted the gel to the same

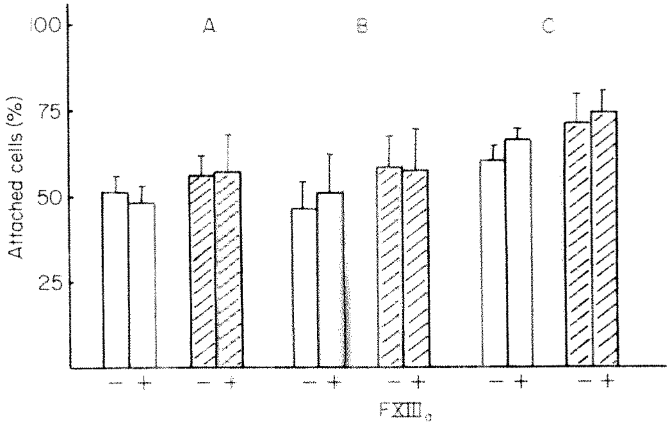

FIGURE 1. Attachment of normal (N) (□) and scleroderma (PSS) (▨) fibroblasts (2×10^5 cells in 500 μl serum free DMEM per well) in 30 min to 10 μg of dried collagen I (A) or III (B) polymers or fibronectin (C), in absence (−) or presence (+) of 1 U/ml of FXIIIa. Each column represents the mean of attachment of five strains of N fibroblasts or of 13 strains of PSS fibroblasts ± the SD.

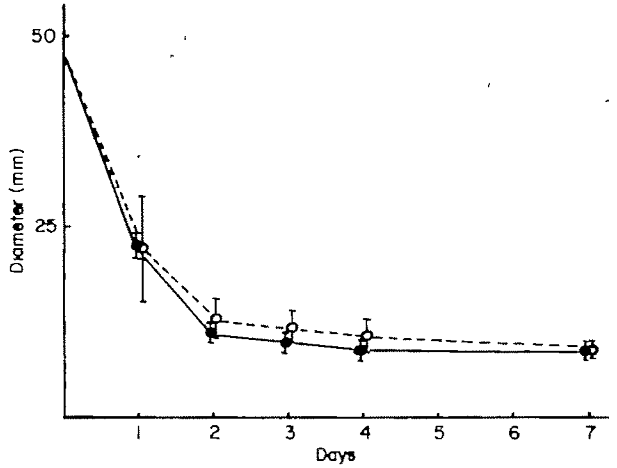

FIGURE 2. Retraction of collagen lattices by N (●——●) and PSS (○ - - - ○) fibroblasts (4×10^4 cells per lattice) determined by measuring the diameter (mm) of the lattice. Each point represents the mean of the measurements performed on three strains of N fibroblasts and of six strains of PSS fibroblasts ± the SD.

extent. Some variability was observed after 1 day of retraction for PSS fibroblasts as shown by the high standard deviation (Fig. 2). FXIIIa (1 U/ml) added during the formation of the lattices did not significantly modify the retraction of the gels for both cell types.

The proliferation capacity of normal and PSS fibroblasts in cell culture was determined in sparse and in 2-day post-confluent cultures. The mean [^{3}H]-thymidine uptake per μg of DNA was not significantly different between the 13 strains of PSS fibroblasts and the six strains of normal fibroblasts. FXIIIa (1 U/ml) displayed variable but not a systematic (increased, unchanged or reduced) effect on fibroblast proliferation (data not shown).

Most of the PSS fibroblast strains at low population doubling level displayed increased collagen synthesis when compared to fibroblasts from the uninvolved skin of a scleroderma patient (patient 1) or to the five strains of skin fibroblasts from normal subjects. In the presence of FXIIIa (1 U/ml), collagen synthesis of PSS fibroblasts was reduced to a level similar to that of normal fibroblasts cultured in the absence of FXIIIa. FXIIIa also inhibited collagen synthesis by normal fibroblasts (Table 1). Non-collagen protein synthesis was increased in PSS fibroblasts as compared to that of normal fibroblasts, and was reduced by FXIII for both cell types (Table 2). The proportion of total protein synthesis accounted for by collagen was higher in PSS fibroblasts than in normal fibroblasts. FXIIIa (1 U/ml) added in the culture medium reduced this proportion (Table 3). FXIIIa did not modify the distribution of the newly synthesized collagen between the soluble form secreted in the medium and the insoluble form deposited in the extracellular matrix in either normal or scleroderma fibroblast strains.

When fibroblasts were included into collagen lattices and labelled with [^{3}H]-proline after 2 days of retraction (lattices fully retracted), collagen synthesis was strongly reduced, although it remained higher for PSS than for normal fibroblasts. The addition of FXIIIa further reduced the biosynthetic activity of both cell types in collagen lattices. Such results are shown in Figure 3 for reticular dermis fibroblasts from the lesion of patient 1 (PSS 1 FR) as compared to those of a healthy region of the same patient (N1 AR). Similar results were obtained with other strains (not shown).

The proportion of degraded newly synthesized collagen was similar for normal and PSS

TABLE 1. Effect of FXIIIa on collagen synthesis

	Patients	Collagen synthesis (10^3 c.p.m./μg DNA) Without FXIIIa	With FXIIIa
N	1 AP	1·01	0·20
	AR	3·43	1·05
	2	33·74	10·78
	3	3·04	1·27
	4	19·49	6·85
	5	6·84	2·87
	6	7·57	3·83
	Mean	10·73	3·84
PSS	1 FP	12·10	3·59
	FR	12·57	4·08
	7 FP	21·92	6·02
	FR	15·53	3·74
	AP	33·53	6·12
	AR	39·85	6·89
	8 FP	73·83	16·72
	FR	61·32	13·74
	AP	53·49	12·79
	AR	56·79	12·85
	9	6·77	2·13
	10	17·60	4·40
	11	7·51	2·82
	Mean	31·75	7·38

Collagen synthesis by normal (N) and scleroderma (PSS) fibroblasts cultured in monolayer on plastic was estimated in absence or presence of 1 U/ml of FXIIIa by labelling with [^{3}H]-proline and measuring peptide bound non-dialyzable [^{3}H]-hydroxyproline in the medium and in the cell layer. For each patient, the synthesis was determined from triplicate dishes with a standard deviation of about 10%. For patients 1, 7 and 8, collagen synthesis was measured in papillary (P) and reticular (R) dermis fibroblasts from abdomen (A) and forearm (F). Histology showed typical lesions of PSS both in abdomen and forearm of patients 7 and 8, and only in forearm of patient 1. Abdomen fibroblasts from patient 1 were considered as non pathological.

fibroblasts whatever the system of cell culture (monolayer or collagen lattice). FXIII increased it significantly in normal and PSS fibroblasts included into a three-dimensional collagen gel (Table 4).

The fibroblasts derived from skin biopsies of patients 1, 7 and 8 were collected before and after treatment by FXIII from the same locations. Labelling with [^{3}H]-proline under identical conditions and at the same passages in subculture was performed. No significant difference in the level of biosynthesis or degradation could be observed between cells collected before and after treatment (data not shown).

TABLE 2. Effect of FXIIIa on non-collagenous protein synthesis

		Non-collagen protein synthesis (10³ c.p.m./μg DNA)	
	Patients	Without FXIIIa	With FXIIIa
N	1 AP	56·90	14·12
	AR	72·04	19·99
	2	107·60	43·85
	3	48·39	25·65
	4	72·25	35·16
	5	46·90	31·68
	6	43·93	28·40
	Mean	64·00	28·41
PSS	1 FP	110·27	40·38
	FR	87·59	37·94
	7 FP	86·07	31·60
	FR	60·30	19·79
	AP	79·05	19·90
	AR	95·67	30·09
	8 FP	179·55	64·79
	FR	149·07	39·81
	AP	139·79	48·55
	AR	141·21	44·73
	9	86·02	34·80
	10	87·26	28·11
	11	71·14	42·26
	Mean	105·61	37·13

Non-collagen protein synthesis by normal (N) and scleroderma (PSS) fibroblasts cultured in monolayer on plastic was estimated by labelling with [^{3}H]-proline and measuring the total non-dialyzable peptide bound radioactivity in the medium and in the cell layer. The radioactivity accounted for by collagen was substracted. The values were obtained from the same labelling experiments as in Table 1.

DISCUSSION

The present study was carried out to determine the mechanism by which FXIII acts in the treatment of scleroderma. The administration of FXIII has been found to increase skin suppleness and improve joint motility in about 50% of the scleroderma patients[7,8] and a similar clinical effect was seen in two of three patients who were treated by FXIII and used in our *in vitro* studies.

As previously described,[4,28] most but not all fibroblast strains derived from the involved skin of scleroderma patients displayed *in vitro* an excessive collagen synthesis as compared with

TABLE 3. Collagen synthesis as percentage of total protein synthesis

	Patients	Collagen/total protein × 100 Without FXIIIa (a)	 With FXIIIa (b)	Ratio $\frac{(b) \times 100}{(a)}$
N	1 AP	0·32	0·25	78
	AR	0·89	0·98	110
	2	7·54	5·50	73
	3	1·18	0·92	78
	4	6·19	4·14	67
	5	2·96	1·75	59
	6	3·58	2·24	63
	Mean	3·24	2·26	75 ± 16
PSS	1 FP	2·15	1·71	79
	FR	2·91	2·10	72
	7 FP	5·75	4·03	70
	FR	5·83	3·99	68
	AP	11·63	7·35	63
	AR	11·31	5·04	44
	8 FP	11.09	5.85	53
	FR	11·09	8·60	78
	AP	9·96	6·01	60
	AR	10·72	6·71	63
	9	1·50	1·15	77
	10	4·32	3·21	74
	11	2·06	1·26	61
	Mean	6·95	4·39	66 ± 10

The proportion (in %) of protein synthesis accounted for by collagen production for normal (N) and scleroderma (PSS) fibroblasts labelled in absence or in presence of FXIIIa (1 U/ml) was calculated from Tables 1 and 2 taking into account the fact that proline is 5·6 times more abundant in collagen than in non-collagen proteins. The last column represents the ratio × 100 between the proportion of protein synthesis accounted for by collagen production in absence of FXIIIa and that obtained in presence of FXIIIa. The mean ± SD of these ratios for the different strains of normal and scleroderma fibroblasts is given.

fibroblasts derived from normal subjects matched for age and sex. On average the production of collagen by scleroderma fibroblasts was three times higher than that of normal fibroblasts or fibroblasts from uninvolved areas of skin in scleroderma.

In the presence of FXIIIa, collagen synthesis by scleroderma cells was reduced to a level comparable to that of untreated normal cells. Non-collagen protein synthesis was also enhanced in scleroderma strains but to a lesser extent, so that the proportion of total protein synthesis accounted for by collagen production was only two times higher in scleroderma than in normal fibroblasts. FXIIIa reduced this percentage to values observed in normal cells. Although an

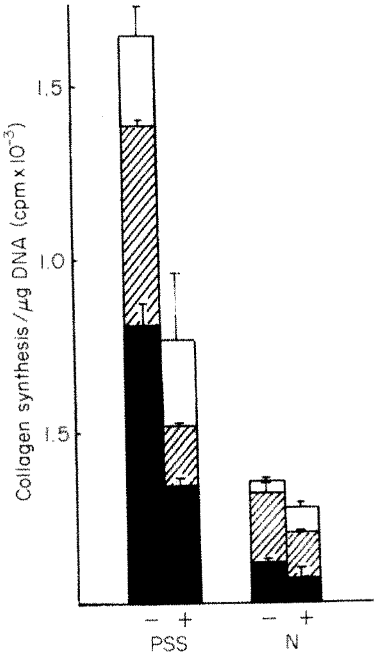

FIGURE 3. Collagen synthesis by scleroderma (PSS: strain 1 FR) and normal (N: strain 1 AR) fibroblasts (2×10^5) included into collagen lattices for 48 h and then labelled for 24 h with [^{3}H]-proline in absence (–) or presence (+) of 1U/ml of FXIIIa. Collagen synthesis is subdivided into insoluble collagen incorporated into the cell layer (■), into soluble and non-dialyzable collagen released in the medium (▨) and into degradation products of collagen (□). Each column represents the mean of the assays performed on three distinct lattices ± SD.

TABLE 4. Effect of FXIIIa on collagen degradation by normal and scleroderma fibroblasts cultured in monolayers or in lattices

	Normal fibroblasts		Scleroderma fibroblasts	
	FXIII	% of degradation	FXIII	% of degradation
Monolayer	–	16 ± 5	–	15 ± 5
	+	19 ± 4 (*n* = 5)	+	20 ± 7 (*n* = 13)
Lattice	–	9 ± 5	–	13 ± 4
	+	28 ± 4 (*n* = 2)	+	30 ± 4 (*n* = 2)

Degradation of newly synthesized collagen by normal and scleroderma fibroblasts cultured in monolayer or within lattice and incubated or not with FXIIIa (1 U/ml) was determined after labelling with [^{3}H]-proline for 24 h by measuring dialyzable peptide-bound [^{3}H]-hydroxyproline in the medium. The results are expressed in % of total [^{3}H]-hydroxyproline recovered in the cell layer and in the medium. Each value represents the mean of the results obtained with *n* different strains ± the SD.

excessive collagen synthesis was previously reported only for fibroblasts derived from reticular dermis,[29] in our experiments the papillary dermis fibroblasts from scleroderma skin also synthesized excessive amounts of collagen. We did not observe any significant difference in terms of biosynthetic activity between the fibroblasts collected before and after treatment of the patients by FXIII. The post-treatment fibroblasts remained sensitive to the addition of FXIIIa in the culture medium. The repression of collagen synthesis by FXIIIa is not specific to scleroderma fibroblasts and is also observed in normal skin fibroblasts, suggesting the involvement of this factor in a more general process of regulation.

FXIII has been shown to promote the attachment of a transformed cell line[19] to collagen I and fibronectin. The adhesive properties of scleroderma fibroblasts to collagen I or III and to fibronectin were similar to those of normal fibroblasts and FXIII did not significantly modify it. The mitogenic effect of FXIIIa on normal and scleroderma fibroblasts was increased in some strains, as previously described for normal and neoplastic fibroblasts,[21,22] and reduced for others. There was no correlation between the patients whose fibroblasts were growth-inhibited by FXIIIa, and those showing a clinical improvement with treatment by FXIII.

When included into fully retracted, three-dimensional heat-reconstituted floating collagen gels, skin fibroblasts underwent a reduction of collagen synthesis of about 80–90%.[30] Scleroderma skin fibroblasts exposed to these conditions displayed the same reduction, apart from one strain (patient 11) with a reduction of 50%. A previous study found a lower reduction of collagen synthesis for scleroderma than for normal fibroblasts and even, in some cases, a stimulation of synthesis in fully retracted collagen lattices.[28] Even when their biosynthesis was repressed in collagen lattices in a proportion similar to that of normal cells, all the scleroderma fibroblasts maintained a higher level of synthesis than normal fibroblasts in the same conditions. The addition of FXIIIa reduced it to a level similar to untreated normal cells as already observed in monolayer culture.

Besides the reduction of collagen synthesis, FXIII also induced an increased degradation of newly synthesized collagen of normal or scleroderma fibroblasts included into collagen lattices. It is known that culturing skin fibroblasts within a free-floating collagen gel induces the expression of the collagenase gene, perhaps through reorganization of the cytoskeleton.[31,32] A potential modification in the mechanism of attachment of the cells to their fibrous support by FXIII might be a factor in this enhanced degradation.

Other hypotheses have been proposed to explain the beneficial role of FXIII in the treatment of scleroderma. Based on work showing an increase in fibronectin in the deep dermis of sclerotic lesions[33] and that describing an induction of cross-linking between collagen and fibronectin by FXIII,[34] Guillevin *et al.*[8] proposed that fibronectin cross-linked to collagen could promote, by its opsonic activity, the degradation of collagen by the reticulo–endothelial system. This mechanism could not be investigated in our *in vitro* conditions and cannot explain the effect of FXIIIa on fibroblast cultures. A stabilization and immobilization of abnormally soluble collagen by FXIII to explain its beneficial effect has also been proposed.[7] However, no modification in the distribution between soluble and insoluble collagen was detected in our scleroderma fibroblasts cultures when incubated in the presence of FXIII.

FXIIIa has an effect on scleroderma and normal fibroblasts and strongly inhibits collagen synthesis and increases the degradation of newly synthesized collagen, reducing the total amount of collagen produced by the cells and incorporated into the tissue. It is likely that these mechanisms contribute to the beneficial effect of FXIII in the treatment of patients with scleroderma.

ACKNOWLEDGMENTS

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Type VII collagen is expressed but anchoring fibrils are defective in dystrophic epidermolysis bullosa inversa

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SUMMARY

A patient with dystrophic epidermolysis bullosa inversa was studied using electron microscopy and indirect immunofluorescence using antibodies to matrix macromolecules of the dermo-epidermal junction zone. There was splitting below the lamina densa with an apparently normal basement membrane, but a lack of intact anchoring fibrils and with a disarranged papillary connective tissue. Indirect immunofluorescence examination with antibodies to type VII collagen, the major structural protein of anchoring fibrils, showed a normal linear staining pattern. Synthesis of type VII collagen which is unable to form stable, resistant anchoring fibrils may be a distinct feature of this subtype of recessive dystrophic epidermolysis bullosa.

Sixteen different subsets of epidermolysis bullosa (EB) have been defined so far using genetic, clinical and ultrastructural criteria.[1,2] The disorders can be placed in three main categories: EB simplex with intraepidermal blistering; junctional EB with lamina lucida blistering; and dystrophic EB with sub-lamina densa blistering. The dystrophic group exhibits clinical and molecular heterogeneity and both dominant and recessive forms contain subgroups. The recessive dystrophic group consists of four subtypes: the localized, the generalized non-mutilating, the generalized mutilating and the inversa types.[1-3]

Dystrophic epidermolysis bullosa inversa is characterized by severe blistering and scarring of the flexures of the skin with milder manifestations on the extremities and a frequent association with extensive involvement of the oesophagus. Only a few patients with this disorder have been described.[3-7] Ultrastructural studies have demonstrated a variable number of poorly formed[7]

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and normal[8] anchoring fibrils. No immunohistological studies using antibodies to basement membrane zone proteins have been reported.

Recent immunohistological and biochemical studies have indicated an absence of type VII collagen, the major structural protein of anchoring fibrils, in the generalized and mutilating type of recessive dystrophic EB.[9–11] This finding supports the earlier ultrastructural studies where no intact anchoring fibrils were found in the clinically uninvolved skin of these patients.[12–14] On the other hand, the localized form of recessive dystrophic EB presents with ultrastructurally distinct anchoring fibrils and with immunoreactive type VII collagen in the patients' skin.[9,10,14] The inversa subgroup may represent a pathogenetically intermediate form of the recessive dystrophic EB. A patient with this form was studied using electron microscopy and immunofluorescence techniques to correlate the ultrastructure of the dermo–epidermal junction zone with the immunohistochemistry of the components of the basement membrane zone.

CASE REPORT

A 19-year-old woman, the second child of healthy unrelated parents, had a history of skin blisters since birth. The blistering was more pronounced in the groins, lower back and abdomen, and neck as well as on the distal parts of the extremities. The blisters were induced by trauma and healed to form atrophic scars and milia. During the last 2 years the patient had several episodes of oesophageal obstruction with upper and lower strictures and multiple oesophageal dilatations have been performed. She also had a history of anal scarring and difficulty with defecation. The patient's parents and brother had no manifestations of a similar skin disorder.

She appeared pale and was underweight (162 cm, 38 kg) and had erosions and blisters in both groins, on the lower abdomen and back (Fig. 1a). Mild to moderate blistering was observed on the neck, axillae, the flexor surface of the knees, elbows and on the hands and feet. Atrophic scars and milia were found in blister-prone areas. The submammary folds were not blistered. The finger- and toe-nails were either dystrophic or lost apart from one finger-nail on the left hand. There was a mild syndactyly of the second and third left toe (Fig. 1c). The patient was unable to protrude her tongue to the teeth and showed relative microstomia with slight recession of the chin. Several teeth that had fractured were capped with an acrylic dental material. X-ray examination of the oesophagus disclosed long strictures (Fig. 1b).

Laboratory investigations indicated a haemoglobin concentration of 10·4 g/l with a haematocrit of 0·31, serum iron 1·7 μmol/l and transferrin 2·9 g/l. Cultures of the skin erosions grew *Staphylococcus aureus*.

METHODS

Skin biopsies

Biopsies were obtained from lesional skin and from clinically uninvolved skin. Part of the uninvolved site was gently rubbed prior to biopsy to induce dermo–epidermal separation. A biopsy specimen from non lesional skin in a predilection site was used for the immunofluorescence studies.

Immunofluorescence studies

Indirect immunofluorescence was performed on 5 μm thick sections cut in a cryostat, using specific monoclonal or polyclonal antibodies to basement membrane zone antigens.[15] The type

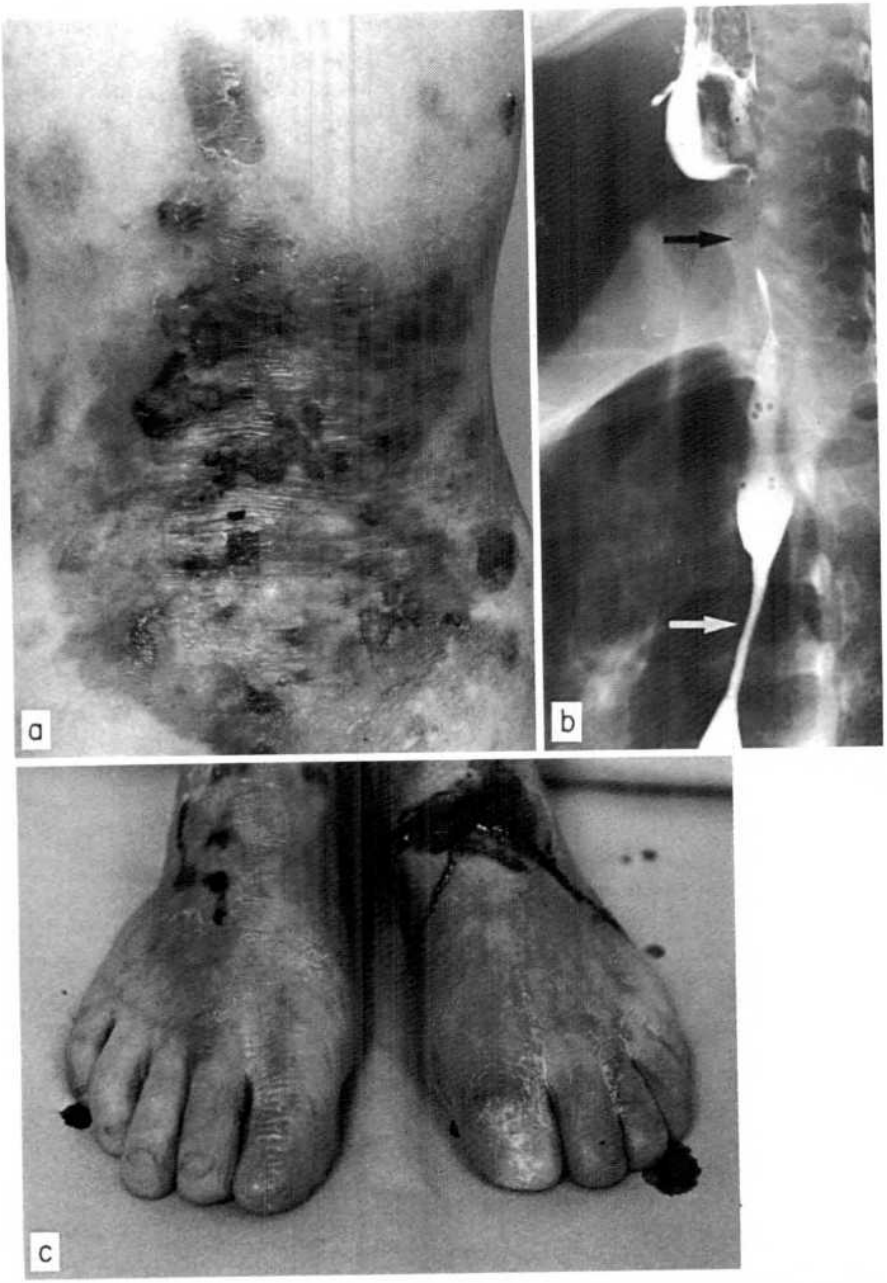

FIGURE 1. (a) Lower back of the patient with erosions and atrophic scars. (b) X-ray of the oesophagus with contrast medium. Note the strictures (arrowed). (c) Dystrophic or missing toe-nails and atrophic skin of feet.

IV collagen antibody was a mouse anti-human monoclonal antibody[16] (gift from Dr Odermatt, Department of Pathology, University of Zurich, Switzerland). The type VII collagen antibody was an affinity-purified polyclonal rabbit antibody to the triple-helical domain of human collagen VII.[17] An EB acquisita serum was used as an internal control to visualize the carboxy terminus of type VII collagen.[18] The laminin antibody was a mouse monoclonal anti-human laminin antibody[19] (Calbiochem, Lucerne, Switzerland). The specificity of each antibody was tested with ELISA, immunoblotting and immunosorbent chromatography. The FITC-labelled second antibodies to mouse, rabbit or human immunoglobulins were obtained from Dakopatts, Glostrup, Denmark. The incubations with the antibodies were carried out for 30 min each at room temperature and the sections then extensively washed. The fluorescence was observed using a Zeiss fluorescence microscope.

Electron microscopy

For ultrastructural studies the three biopsies were fixed first with 2·5% glutaraldehyde in phosphate buffer and stained with 2% osmium tetroxide, processed in EPON LX-112, and stained with uranyl acetate and lead citrate. The ultra-thin sections were observed with a Jeol JEM-1200 EX microscope.

RESULTS

Light microscopy

Routine light microscopy of the lesional skin at the edge of a fresh natural blister showed a subepidermal blister with normal epidermis as the roof. The bottom of the blister was formed by a non-inflamed papillary dermis.

Indirect immunofluorescence

Immunofluorescence studies with antibodies to type VII and type IV collagen (Fig. 2) and laminin (not shown) revealed a linear fluorescence at the dermo–epidermal junction zone similar to that seen in normal skin. In a blistered area, all the antigens were localized to the blister roof, a finding consistent with sub-lamina densa blistering. An additional control stained with serum of an EB acquista patient (containing circulating antibodies to the carboxy terminus of type VII collagen) yielded an identical pattern of fluorescence. Antibodies to type IV collagen and laminin also stained the basement membranes of the blood vessels.

Electron microscopy

Electron microscopic studies were performed on a fresh blister and on clinically uninvolved skin from a non-predilection area. The third biopsy was from clinically uninvolved skin in the axilla. In the blister there was a split below the basement membrane (Fig. 3). The lamina densa and lamina lucida appeared normal with basal keratinocytes and hemidesmosomes at the roof of the blister.

In the specimen obtained from non-rubbed, healthy looking skin from a non-predilection area, the hemidesmosomes, lamina densa and lamina lucida seemed to be normal. However, there were no normal anchoring fibrils and long elastic microfibrils appeared to be absent (Fig. 4). The space was filled with an amorphous substance crossed by haphazardly arranged collagen fibres. Irregular duplications of the lamina densa were seen, and rudimentary anchoring fibrils without distinct crossbanding were found focally, as were very short microfibrils.

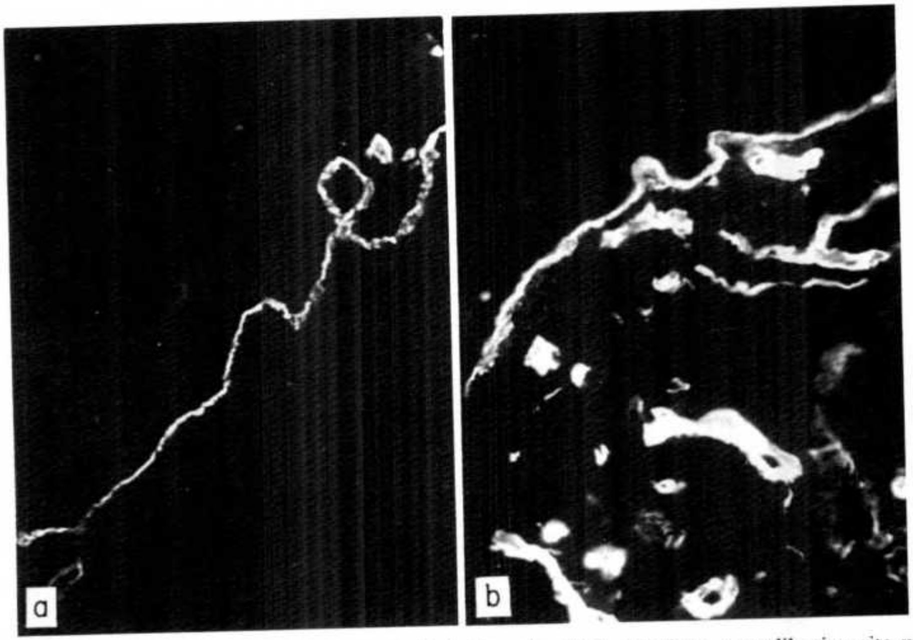

FIGURE 2. Indirect immunofluorescence of clinically uninvolved skin from a predilection site with antibodies to type VII collagen (a) and type IV collagen (b). There is a continuous linear fluorescent staining at the dermo–epidermal junction zone with both antibodies. Type IV collagen also stains the vascular basement membrane.

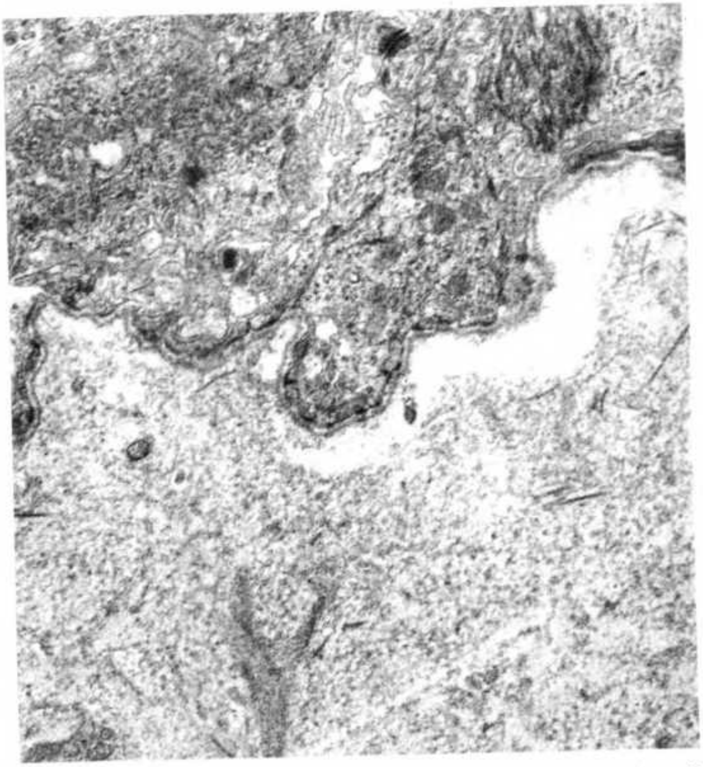

FIGURE 3. Electron micrograph of the biopsy from the edge of a fresh blister showing splitting below the lamina densa and disarranged connective tissue. × 10 000.

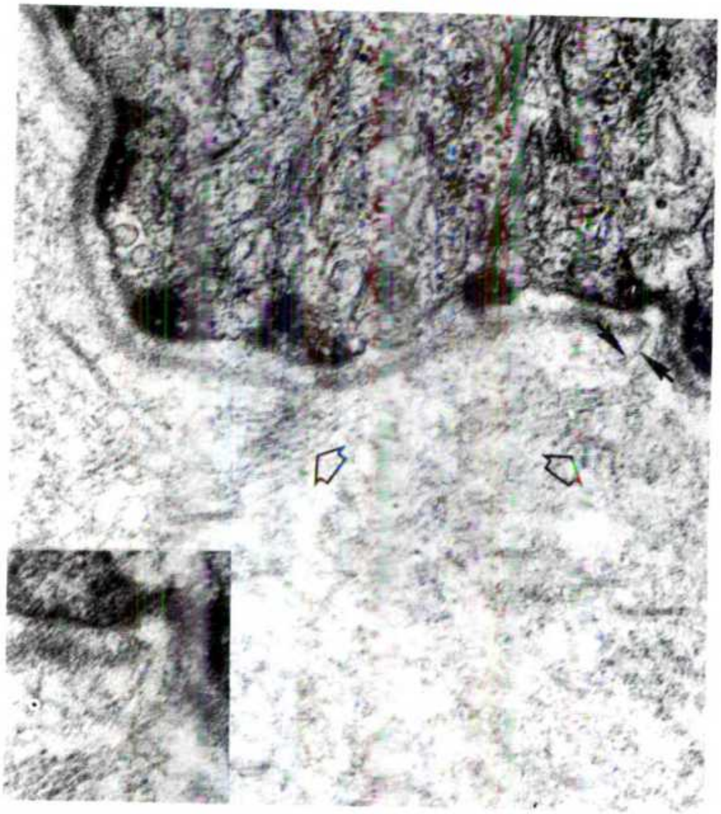

FIGURE 4. Electron micrograph of a biopsy taken from clinically uninvolved skin in a non-predilection site. The area below the lamina densa is devoid of normal anchoring fibrils. A rudimentary anchoring fibril (arrows) is depicted in the inset (× 60 520). Normal microfibril bundles are indicated with open arrows (× 38 000).

The clinically uninvolved, non-rubbed skin from a predilection site near the axilla was severely affected. There were several small vacuoles beneath the lamina densa and the area below this was oedematous with amorphous material. In addition, disrupted and partially phagocytized red blood cells were observed. Anchoring fibrils were only occasionally found and displayed corkscrew-like thin spirals.

DISCUSSION

In our case of dystrophic EB inversa, all characteristic manifestations of this subtype[3,7] were present apart from the keratitis. Ultrastructural studies on the biopsies of skin confirmed the diagnosis of dystrophic EB. Blistering occurred below the basement membrane and degenerated sub-lamina densa tissue with rudimentary anchoring fibrils were seen even in the clinically normal skin. In the predilection sites the sub-lamina densa area around blisters and scars was more affected.

Recently, electron microscopical studies were performed on four patients with dystrophic EB inversa.[7] In comparison with our cases they found a higher number and more distinct anchoring fibrils in the predilection sites. The authors interpreted their finding as comparable with the milder forms of generalized non-mutilating recessive dystrophic EB. Earlier studies described five patients with EB inversa, of which two had rudimentary and three normal anchoring fibrils[8] but other studies[4,6] have not examined the presence of anchoring fibrils. We found an absence of anchoring fibrils together with disarrangement of the sub-lamina densa area. These electron microscopic studies suggest that the ultrastructural appearance of anchoring fibrils varies from patient to patient. This may be due to different biopsy sites or represent heterogeneity within the EB subtype.

Indirect immunofluorescence staining with type IV collagen and laminin antibodies showed a normal staining pattern in our patient. This is in accordance with the ultrastructural finding of an intact lamina densa and lamina lucida. Both antigens were localized to the blister roof, which agrees with dystrophic blistering. However, there was normal linear fluorescence with type VII collagen antibodies, although no distinct, crossbanded anchoring fibrils were seen on electron microscopy. This suggests the presence of type VII collagen molecules that were not assembled into the appropriate supra-structure. This may be due to a defect in the biosynthesis of type VII collagen that prevents normal supramolecular assembly and the formation of stable anchoring fibrils. Secondly, normal type VII collagen and anchoring fibrils may be proteolytically degraded, e.g. by the upregulated mutant collagenase as suggested by Bauer *et al.*[20] The positive immunofluorescence would then result from the reaction of the antibodies with persisting immunoreactive fragments. Thirdly, other putatively abnormal interactions of the anchoring structures, e.g. abnormal molecular interaction between type VII collagen and lamina densa components, such as type IV collagen, heparan sulphate proteoglycan or entactin, may render the anchoring fibrils unstable and susceptible to proteolytic degradation.

The presence of type VII collagen and the absence of intact anchoring fibrils in EB inversa suggest that this subtype is a distinct genetic disorder in the recessive dystrophic EB group. The localized recessive dystrophic EB exhibits both normal anchoring fibrils[1] and positive type VII collagen immunofluorescence[9,10] whereas in the majority of the patients with generalized mutilating disease neither anchoring fibrils[1,8,11,12,14] nor type VII collagen[9–11] can be demonstrated. However, a recent study[21] indicated that the carboxy terminus of type VII collagen may be conserved in some cases of generalized mutilating dystrophic EB. These studies on the expression of type VII collagen provide further evidence that the different subtypes of recessive dystrophic EB represent different gene defects and not more or less severe variants of the same genetic disorder.

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Intraepidermal leakage of plasma proteins after tape stripping of normal skin and uninvolved psoriatic skin

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SUMMARY

Following tape stripping of normal skin and uninvolved psoriatic skin, there was an early leakage of plasma proteins such as fibrinogen, fibronectin and immunoglobulins. This was accompanied by migration of mononuclear and polymorphonuclear cells into the epidermis. Both events appeared earlier, were more pronounced and lasted longer in psoriatic subjects than in controls.

Research into psoriasis has mainly involved the study of epidermal alterations such as hyperproliferation and abnormal differentiation.[1-3] However, morphological studies[4] have demonstrated that psoriasis is also characterized by vascular alterations such as dilatation of capillary loops within the lesions. At the ultrastructural level, the intradermal papillary vessels have many bridged fenestrations,[5] which are absent in normal skin.[6] These morphological changes are detectable even before the development of epidermal hyperplasia. Under successful Goeckerman and PUVA treatment, the capillary loops return to normal before there is a decrease in the labelling index.[7] Functional evidence of decreased capillary resistance was also shown in the uninvolved skin of psoriasis.[8]

Previous studies using immunofluorescence techniques revealed the presence of several plasma proteins within the involved psoriatic epidermis.[9-12] Fibronectin, fibrinogen, and IgG deposits were observed just above the papillae. Such deposits were not detected in normal or uninvolved psoriatic epidermis. The pattern of staining above the papillae suggested that the plasma proteins were leaking from the capillaries into the extravascular space, and that an increased vascular permeability of upper dermal capillaries could be one of the initial events triggering hyperproliferation and abnormal differentiation of psoriatic epidermis. This increased vascular permeability could for example, allow an increase in diffusion of nutrients toward the epidermis and the passage of plasma growth factors into the epithelium.

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The leakage of plasma proteins into the epidermis following tape stripping of uninvolved psoriatic skin was studied. This model[13,14] allows determination of the changes prior to the development of a psoriatic lesion. Lesions induced by tape stripping are similar to spontaneous pinpoint lesions.[15,16] This study followed early events after tape stripping as it was shown that leakage and migration occur as early as 2 min after stripping.[17]

METHODS

Patients

The study included five male psoriatic patients (age range 19–54 years) and four control subjects who were sex- and age-matched healthy volunteers.

Methods

Stripping of the stratum corneum was performed with an adhesive tape (Tesa film®). Thirty to forty strippings were required to obtain a smooth but non-oozing epidermal surface. Strippings were performed on 2 × 8 cm areas of uninvolved forearm or back skin in patients with active psoriasis ($n = 5$) and in normal controls ($n = 4$). In the psoriatic patients, the tape-stripped skin was at least 10 cm away from a lesion. Skin biopsies were taken from each patient at intervals of 0, 2·5 min, 15 min, 1 h, 24 h and 1 week after stripping.

Histology

Serial sections of the skin biopsies were performed and stained with hematoxylin and eosin.

Direct immunofluorescence

The biopsies were immediately frozen in tissue TEK II (3M) and cryostat sections (4 μm thick) were cut and then incubated, as described previously,[10] with fluorescein-conjugated goat antibodies directed either against human fibrinogen, fibronectin or immunoglobulin G (Cappel Laboratories, Cochranville, U.S.A.).

RESULTS

Clinical observations

One or 2 weeks following tape stripping a Koebner phenomenon was observed in two of the five psoriatic patients. There were red, inflammatory plaques in the tape-stripped area. The other three psoriatic patients as well as the four normal controls showed complete healing after a 2-week period.

Histology

Immediately following the stripping procedure there were no significant changes seen. Biopsies taken 2·5 min after tape stripping showed a moderate cellular infiltrate in the dermis in both psoriatic and control subjects. In the control skin, most of the infiltrate was composed of mononuclear cells. In the tape-stripped uninvolved psoriatic skin, the cellular infiltration was more pronounced and was composed of mononuclear and polymorphonuclear cells. Fifteen minutes, 1 h and 24 h after stripping, the polymorphonuclear infiltration was more intense than at 2·5 min and mainly located around the upper dermal vessels. The cells had also migrated toward the epidermis and their density was again higher in uninvolved psoriatic skin than in the controls. After 1 week, some areas of parakeratosis could be already observed in tape-stripped

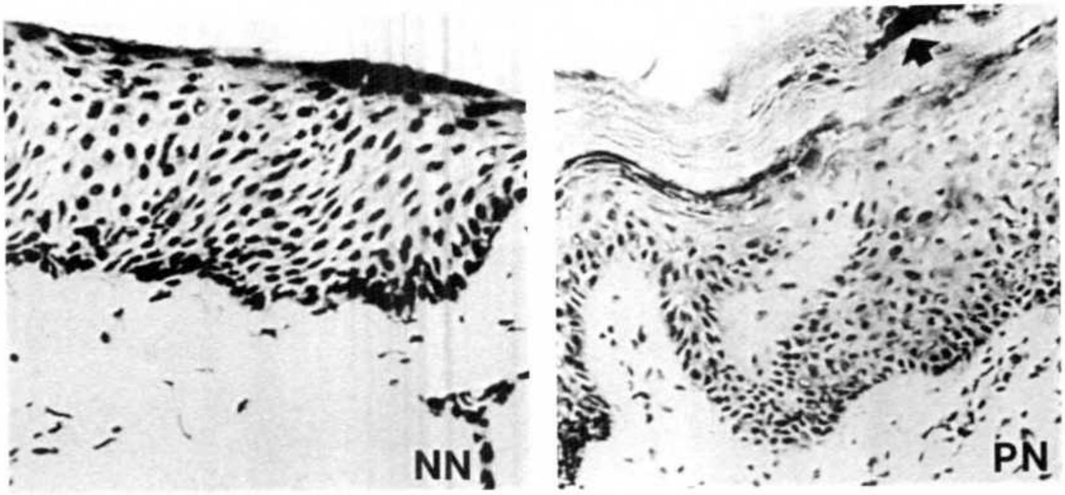

FIGURE 1. Biopsies taken 1 week after stripping from control skin (NN) and uninvolved psoriatic skin (PN). Note the accumulation of PMN in the stratum corneum of PN (arrow) (× 240).

uninvolved psoriatic epidermis and accumulations of polymorphonuclear cells (PMN) in the stratum corneum, very similar to the Munro Sabouraud micro-abscesses, were observed in some sections of the uninvolved psoriatic skin (Fig. 1). This phenomenon, which preceded the appearance of clinical lesions, was never observed in normal control skin. The dermal infiltrate disappeared after 1 week in both the psoriatics and controls.

Immunofluorescence

Biopsies taken immmediately after the stripping procedure appeared normal as previously observed (M.Darmon, unpublished results).

Fibrinogen. Biopsies taken from psoriatic patients 2·5 min after tape stripping when stained with FITC-conjugated anti-fibrinogen serum, showed fibrillar and punctate pericellular staining in the dermal papillae (Fig. 2). A focal staining was also detected in the epidermis above

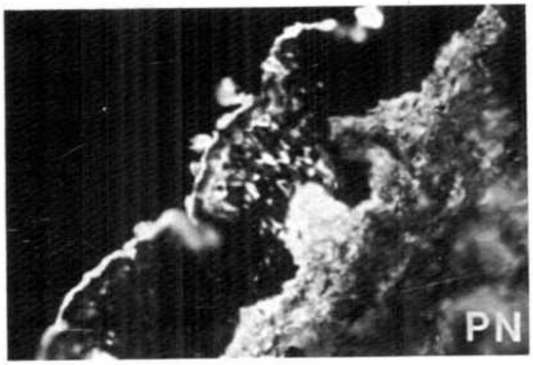

FIGURE 2. Immunofluorescence staining with FITC- conjugated anti-fibrinogen antiserum of a biopsy taken 2·5 min after tape stripping of uninvolved psoriatic epidermis (PN). Note the leakage immediately above the papilla. (× 100).

these dermal papillae. In biopsies taken from normal individuals 2·5 min after stripping, the staining was negative. One hour after stripping (Fig. 3), the fibrinogen pattern was similar in uninvolved psoriatic skin and in controls, consisting of alternating positive (above the papillae) and negative (in epidermal crests) zones. One week after stripping the psoriatic skin stained weakly with the anti-fibrinogen antibody at the granular layer but staining was more marked in the stratum corneum, mainly in the micro-abscesses. This staining was more pronounced in

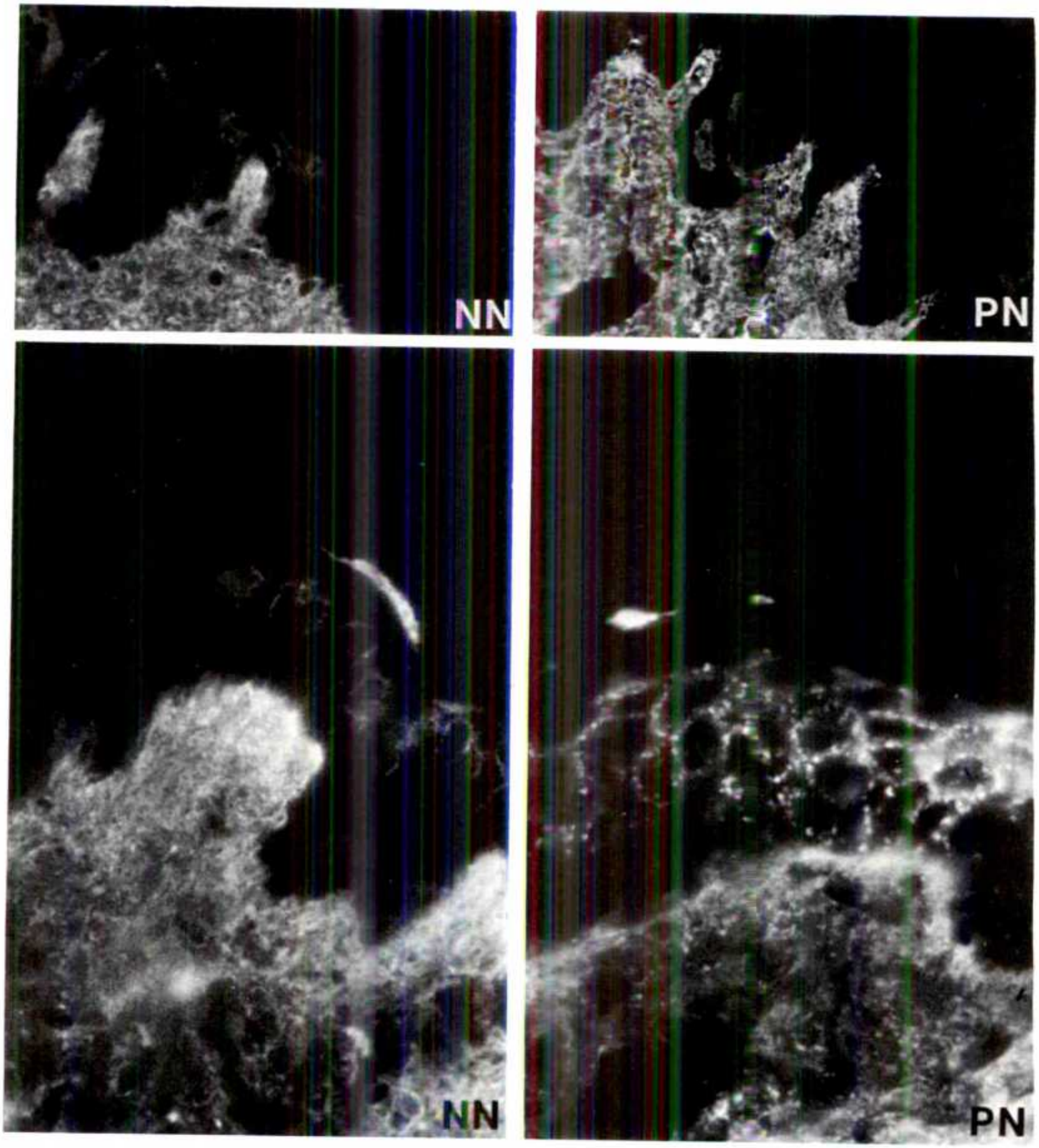

FIGURE 3. Immunofluorescence staining with FITC conjugated anti-fibrinogen antiserum of biopsies taken 1 h after stripping. Note the pericellular staining in the epidermis mainly located above the papillae, more evident in uninvolved psoriatic skin in (PN) than in control skin (NN) (× 100 and × 210 respectively).

patients who developed the Koebner phenomenon. There was no staining seen for fibrinogen in the controls at this time.

Fibronectin. Biopsies taken 2·5 min after stripping showed fibrillar staining in the dermis at the level of the papillae and along the basement membrane in both psoriatic and normal subjects.

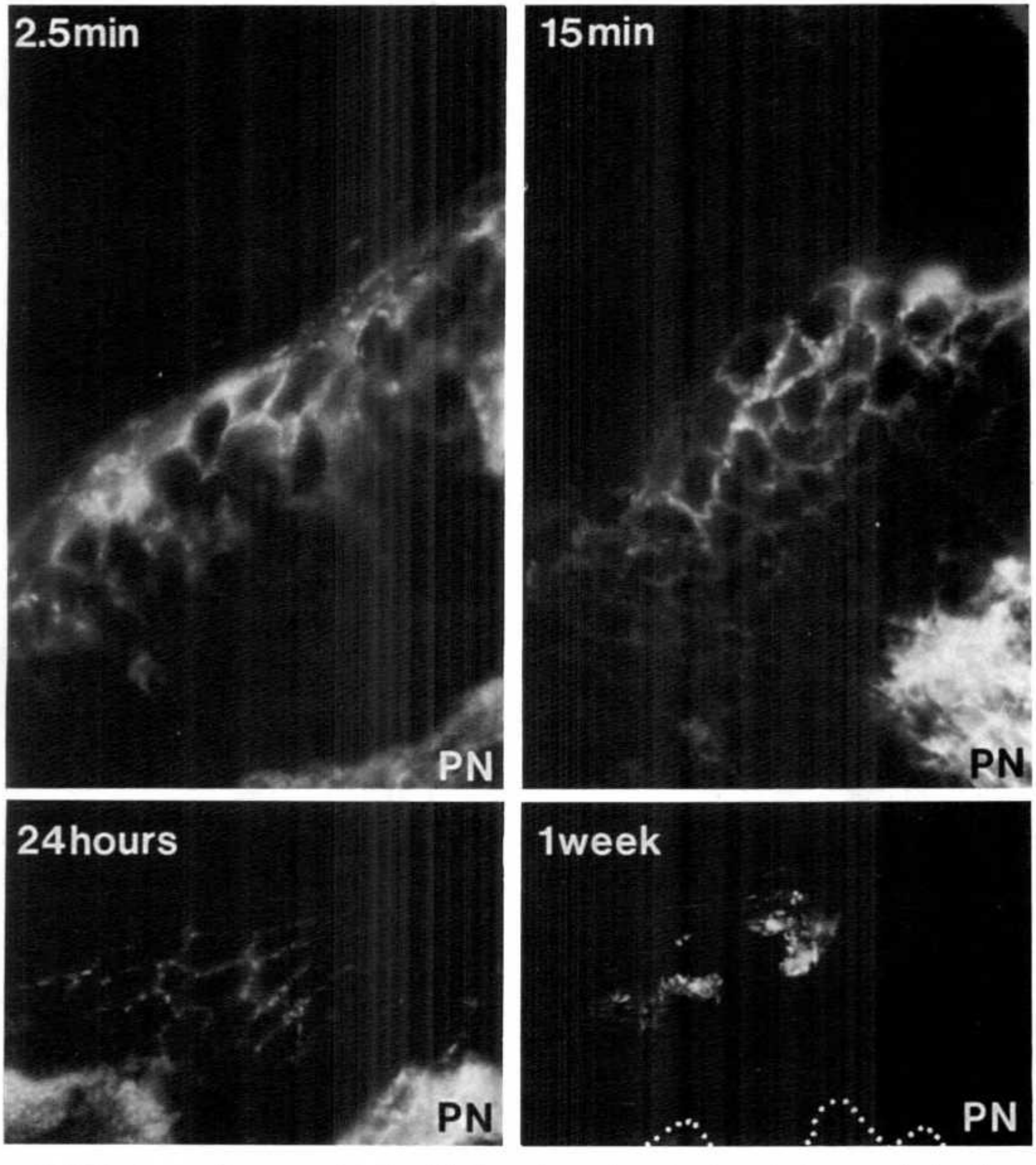

FIGURE 4. Immunofluorescence detection of IgG 2·5 min, 15 min, 24 h and 1 week after tape stripping of uninvolved psoriatic skin. (a) After 2·5 min; diffuse deposits in the dermis (below) and in the upper epidermis. (b) After 15 min; granular and pemphigus foliaceus-like deposits in the upper layers of the epidermis. (c) After 24 h; the same type of intraepidermal immune deposits. (d) After 1 week; foci of immune deposits in the stratum corneum.

Fifteen minutes after stripping, the staining reached the epidermis only in the case of psoriatic subjects. After 1 h, this type of epidermal labelling was found in both psoriatics and controls. Twenty-four hours after stripping, focal deposits of fibronectin were observed at the level of granular layers only in the patients who developed typical psoriatic lesions.

Immunoglobulins. Diffuse IgG deposits in the dermis and in some cases in the epidermis (Fig. 4) were observed in biopsies taken 2·5 min after stripping in both the psoriatic and control subjects. After 15 min, the staining was detected in the upper part of the epidermis of psoriatics with granular and pemphigus foliaceous type pattern and it was always more pronounced above the dermal papillae. In controls, after 15 min, some focal epidermal staining of this type was observed. After 24 h, small foci of marked fluorescence were observed in the stratum corneum of the psoriatic epidermis. After 1 week, the staining was still marked and was located in the stratum granulosum and corneum. In the controls there was no staining in the epidermis at 24 h and after 1 week.

DISCUSSION

In this study, it was found that very shortly after tape stripping of both normal and psoriatic skin, mononuclear cells, polymorphonuclear cells and plasma proteins such as fibrinogen, fibronectin and IgGs extravasated from the capillaries of the upper dermis into the epidermis. This very early diffusion of plasma proteins correlates with an early passage of Langerhans cells[17] and lymphocytes[18] following tape stripping. However, in our study the extravasation appeared earlier, was slightly more pronounced and lasted longer in the psoriatic subjects than in the controls. This suggests an increased fragility of psoriatic capillaries which may be associated with the morphological modifications of the microvasculature already described.[4–7] Also immunotherapy with interleukin 2, which can induce extravasation of plasma proteins, results in the exacerbation of lesions in patients with psoriasis.[19]

Although our study stresses the existence of a vascular defect in psoriasis, vascular leakage cannot be claimed to be the only aetiological factor of psoriatic lesions. The epidermis of normal subjects did not respond to tape stripping by developing psoriasiform lesions, even though, as with psoriatic epidermis there was significant diffusion of plasma proteins and cellular infiltration. The increased vascular leakage provoked by tape stripping of uninvolved psoriatic skin did not lead to a Koebner phenomenon in all cases, the frequency being similar to that found in previous studies.[14,15,17,20] It is likely that there is an intrinsic defect in the keratinocytes in psoriasis which becomes revealed with the passage of growth factors as seen following tape stripping.

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Incidence of non-melanoma skin cancer in West Glamorgan, South Wales

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SUMMARY

A population-based epidemiological study of non-melanoma skin cancer incidence was carried out over 6 months in an area of South Wales. A much higher incidence than expected of non-melanoma skin cancer was found with crude annual rates per 100000 population of 137·7 for basal cell carcinomas and 35·8 for squamous cell carcinomas, giving a combined rate of 173·5. When age was adjusted to the standardized world population the corresponding rates were 83·1, 19·0 and 102·1. Males were affected more than females and the ratio of basal cell carcinomas to squamous cell carcinomas was 4:1. These figures are at least 60% higher than previous estimates of the incidence of non-melanoma skin cancer in the U.K. There may be a higher incidence in this area because of the indigenous Celtic population, but there is almost certainly a substantial under-recording of non-melanoma skin cancers throughout the U.K.

An accurate assessment of the incidence of non-melanoma skin cancer (NMSC) in this country has been impossible. It is generally accepted that they are under-recorded[1] and there have been no epidemiological studies of the incidence of skin cancer in the U.K. It is becoming important to obtain data on the incidence for NMSC to monitor the possible effects of increasing amount of ultraviolet light reaching the earth's surface as a result of the depletion of the ozone layer.[2,3] A study in the U.S. has suggested that there may be a 10% increase in incidence of NMSC over a 10-year period that is unrelated to the ozone layer depletion.[4] Furthermore, the number of NMSC is likely to increase as a result of an increasingly elderly population. Although NMSCs are usually regarded as being relatively easy to treat and diagnose, in view of their increasing frequency they are becoming an important economic and health problem for this country.

We report the results of a 6-month epidemiological survey of all cases of basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) in a defined population in South Wales (West Glamorgan).

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METHODS

The study population was the resident population of West Glamorgan (363 200). All clinicians in relevant specialities (dermatologists, radiotherapists, geriatricians and surgeons) agreed to register their patients with NMSC presenting for the first time between 1 May 1988 and 30 October 1988. The pathologists supplied details of all biopsy specimens with a diagnosis of SCC or BCC. The information received was stored on computer, using a simple database program. Care was taken to avoid multiple registrations from different departments. Local GPs were not informed of this study, so as not to influence referral patterns. The information received included name, address, date of birth, sex, site, clinical diagnosis, whether or not a biopsy specimen was taken and details of histology. Only those with a diagnosis of BCC and SCC were included. Numbers of patients rather than numbers of cancers were counted; therefore, those patients with multiple skin cancers were counted as one case. Recurrent tumours were also excluded.

Statistics

Age and sex incidence rates were calculated using the standard 5-year age divisions (20–24, 25–29, 30–45, etc.). Adjustment for age and standard error were calculated by using the world standardized population, giving a means of comparing incidence figures between different populations.[1]

RESULTS

A total of 315 NMSC were registered in the 6-month period, giving a crude incidence rate of 173·5 per 100000 per annum. The age-specific incidence rates, crude rates and world population standardized rates are shown in Table 1. There were a total of 181 males (crude incidence rate per annum 206·4 per 100000) and 134 females (crude incidence rate 142·6 per 100000 per annum), with an age range of 26–92 years. The age standardized annual rate per 100000 population was 143·9 for males and 60·3 for females, giving a combined rate of 102·1.

As expected, the highest rates were in the very elderly with a yearly incidence for all NMSCs in males and females of 1248 per 100000 in the 85+ age group. Men in general had a higher incidence, reaching 1566 for the 80–84 age group and 1371 for the 85+ age group. The ratio of BCC to SCC was approximately 4:1 overall, but varied according to age. The younger age groups tended to have a higher ratio, with the 50–55 age group having an 8:1 ratio compared with a ratio of 2:1 in the over 80s.

Information on the site of the lesion was obtained in 257 cases. The face and neck were the commonest sites for both SCC (66%) and BCC (81%). Men had a comparatively higher number on the face, in particular on the pinna of the ear, and women had a much higher incidence on the legs, with eight women having SCCs on the legs, compared to one man. Table 2 shows the breakdown of numbers of patients, according to site, sex and type of skin cancer.

Histological confirmation of the diagnosis was made in 74% of BCCs and 98·5% of SCCs.

DISCUSSION

This study has confirmed that skin cancers are the most common cancers, with a crude incidence rate of 173·5 per 100000 per annum. This is much higher than any previous estimate of skin cancers in the U.K. The annual registration rate for 1984, according to the latest figures

TABLE 1. Age-specific, crude and world age standardized incident rates per 100000 population according to sex and type of NMSC (total numbers shown in brackets)

	Male			Female			Both sexes		
Age	BCC	SCC	Total	BCC	SCC	Total	BCC	SCC	Total
25–29	0	0	0	15·6 (1)	0	15·6 (1)	7·8 (1)	0	7·8 (1)
30–34	17·9 (1)	0	17·9 (1)	18·2 (1)	0	18·2 (1)	18·0 (2)	0	18·0 (2)
35–39	49·5 (3)	0	49·5 (3)	65·2 (4)	0	65·2 (4)	57·4 (7)	0	57·4 (7)
40–44	83·9 (5)	0	83·9 (5)	33·9 (2)	0	33·9 (2)	58·6 (7)	0	58·6 (7)
45–49	155·6 (8)	0	155·6 (8)	97·6 (5)	0	97·6 (5)	126·6 (13)	0	126·6 (13)
50–54	261·7 (13)	20·1 (1)	281·8 (14)	59·7 (3)	19·9 (1)	79·6 (4)	160·2 (16)	20·2 (2)	180·2 (18)
55–59	280·4 (14)	80·1 (4)	360·5 (18)	116·4 (6)	0	116·4 (6)	197·2 (20)	39·4 (4)	236·6 (24)
60–64	332·1 (17)	117·2 (6)	449·3 (23)	89·8 (5)	35·9 (2)	125·7 (7)	205·8 (22)	74·8 (8)	280·7 (30)
65–69	727·7 (33)	88·2 (4)	815·9 (37)	219·8 (12)	36·6 (2)	256·4 (14)	350·2 (35)	60·0 (6)	510·3 (51)
70–74	432·3 (18)	207·0 (7)	739·3 (25)	255·0 (12)	0	255·0 (12)	450·2 (45)	86·5 (7)	457·5 (37)
75–79	761·4 (18)	84·6 (2)	846·0 (17)	529·4 (21)	277·3 (11)	806·7 (32)	616·0 (39)	205·3 (13)	821·4 (52)
80–84	988·5 (12)	577·6 (7)	1566·1 (19)	549·0 (15)	292·8 (8)	841·8 (23)	684·3 (27)	380·2 (15)	1064·5 (42)
85+	685·5 (4)	685·5 (4)	1371·0 (8)	895·4 (17)	316·0 (6)	1211·4 (23)	846·1 (21)	402·9 (10)	1249·0 (31)
Total all ages	166·5 (146)	39·9 (35)	206·4 (181)	110·7 (104)	31·9 (30)	142·6 (134)	137·7 (250)	35·8 (65)	173·5 (315)
World standardized (standard error)	112·2 (9·1)	31·7 (4·5)	143·9 (10)	54·1 (5·8)	6·2 (1·28)	60·3 (12·1)	83·1 (7·3)	19·0 (2·4)	102·1 (11·8)

TABLE 2. Numbers of patients with BCC and SCC according to site and sex (percentage of totals in parentheses)

	Head and neck			Arm			Leg			Trunk			All sites		
	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total	Male	Female	Total
SCC	22	15	37 (66%)	2	3	5 (9%)	1	8	9 (16%)	2	3	5 (9%)	27	29	56
BCC	84	79	163 (81%)	0	1	1 (0·5%)	4	4	8 (4%)	26	3	29 (14·5%)	114	87	201
Total NMSC	106	94	200 (78%)	2	4	6 (2%)	5	12	17 (7%)	28	6	34 (13%)	141	116	257

published by the Welsh Cancer Registry for this area, is 67·5, which compares with a figure of 48·3 for the rest of Wales and 43 for England and Wales together[5]. West Glamorgan has consistently had the highest reported rates of NMSC in Wales and one of the highest in the U.K. The Cancer Registry figure is, however, only 39% of the observed figure in 1988, the difference being almost certainly due to under-recording.

The figures obtained for incidence rates in West Glamorgan in this study are probably reasonably accurate. Very few, if any, skin cancers are treated by local general practitioners and if any were treated, they would almost certainly be excised and sent for histology and therefore included in this study. The combined registration from clinicians and pathology laboratories meant that the chance of a NMSC being missed was small. Most of the diagnoses were confirmed histologically and of those that were not, the diagnosis was made clinically, almost always by an experienced Consultant who was either a dermatologist or a radiotherapist. As there are no other figures with which to compare these incident figures within the U.K., apart from Cancer Registry figures, it is impossible to be certain whether or not this type of incidence rate would be seen elsewhere in the U.K. Extending the crude rate for this population to the whole of England and Wales would suggest that there would be approximately 100000 new cases of NMSC every year.

The method used could be criticized on the grounds that the rates are not true incidence figures but represent referral rates for specialist treatment. The same criticism, however, applies to all Cancer Registry figures as they are all dependent upon referrals for treatment. The method is similar to that used in one of the largest epidemiological studies of NMSC,[6] where over a 6-month period new cases presenting to clinicians and pathologists in various geographical locations in the U.S.A. were registered. The total numbers of skin cancers observed in this study are relatively small and care should therefore be taken in interpreting the age-specific incidence rates in particular. They do, however, accord with other much larger previously published studies in that the rates continue to rise with age and are commoner in men than in women. The ratio of BCC to SCC is also comparable to other studies.

Ideally, histological confirmation should be obtained on all cases but, in practice, many small superficial BCCs are treated by non-surgical methods such as cryotherapy or radiotherapy without histological control, particularly in the elderly age group. It may be argued that this 6-month period was unrepresentative, but there is no reason to suspect that the referral rates for diagnosis and treatment of non-melanoma skin cancers vary with the time of year.

There have been few studies in the rest of the world on the incidence of NMSC. A linear relationship between latitude and skin cancer incidence has been found, the highest rates being in white Australians. The recent study from Victoria in Australia, in which the details of 253 patients with NMSC were described, showed a yearly incidence of 823 skin cancers per 100 000 population, with a world standardized rate of 555 per 100 000.[7] This compares with a large study in the U.S.A. showing an age-standardized rate of 233 per 100 000 population in 1978.[8]

The rate of skin cancers seen in this study was higher than one would expect for the latitude. There are few figures with which to compare this rate, but studies in Europe at roughly comparable latitudes showed a much lower incidence rate. Surveys in Switzerland,[9] Belgium[10] and Denmark[11] showed rates varying between 25 and 50% of those in the current survey.

A study in the U.K. from Bristol suggested that the Cancer Registry in that area under-estimated the true incidence of non-melanoma cancer by at least 20–32%.[12] If this were the case, then this would suggest an incidence rate of about 60–70 cases per 100000, which again is far lower than the actual rate in this study.

Possible reasons for a higher rate of skin cancers in this area would include the genetic pool, in that most of the population of West Glamorgan are of Celtic origin. This is largely an industrial area, although there are farming communities. Some of these cancers may have been caused by occupational carcinogens, although in most cases this seems unlikely in view of the large prevalence of BCCs over SCCs and the site distribution of the lesions.

In view of the discrepancy between the Cancer Registry figures and the incident figures obtained in this study, there is an urgent need to develop a comprehensive reporting system to obtain a more accurate assessment of NMSC rates in the United Kingdom. Any future changes as a result of depletion of the atmospheric ozone could then be monitored.

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Recombinant interferon α2a is effective in the treatment of discoid and subacute cutaneous lupus erythematosus

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SUMMARY

Ten patients suffering from either discoid lupus erythematosus (DLE) or subacute cutaneous lupus erythematosus (SCLE) were treated with interferon α2a. Eight received low or intermediate doses ($18–45 \times 10^6$ U/week) for a short period of time (4–8 weeks), with marked improvement of skin lesions in six, an exacerbation in one patient and no change in the other. Two patients with SCLE received high doses ($100–120 \times 10^6$ U/week) over 12 weeks, with complete clearing of the lesions in one and a marked improvement in the other. The responses were of short duration and within a few weeks of stopping treatment all who had improved or cleared relapsed. The side-effects in all the patients were fever and a flu-like syndrome which necessitated a reduction of the dose in one case. In two patients there were increases in the liver enzyme levels, but no haematological toxicity was noted.

Interferon alpha (IFN-α) has produced an improvement in the clinical condition of patients with autoimmune multiple sclerosis,[1] rheumatoid arthritis[2] and immune thrombocytopenic purpura.[3,4] To our knowledge, there has been no study of the efficacy of IFN-α in the treatment of lupus erythematosus (LE). The results are reported of a therapeutic trial using low doses of IFN-α in patients with discoid LE (DLE) and subacute cutaneous LE (SCLE).

METHODS

Patients

Ten patients with discoid (DLE) or a subacute cutaneous (SCLE) lupus erythematosus gave informed consent to participate in the study. The clinical data concerning the 10 patients are listed in Table 1.

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TABLE 1. Clinical data of patients

Patient	Sex/ Age	Diagnosis	Duration (years)	Previous treatments*
1	F/42	DLE	4	Hydroxychloroquine (−) Topical corticosteroids (+)
2	M/58	DLE	15	Hydroxychloroquine (+) Thalidomide (+)
3	F/34	DLE	2	Hydroxychloroquine (+)
4	M/39	DLE	5	Hydroxychloroquine (−)
5	M/55	DLE	1	Nil
6	M/50	DLE	3	Hydroxychloroquine (+)
7	F/52	SCLE	5	Hydroxychloroquine (ci) Thalidomide (ci) Systemic corticosteroids (ci)
8	F/36	SCLE	4	Hydroxychloroquine (−) Thalidomide (+) Systemic corticosteroids (−)
9	M/26	SCLE	1	Nil
10	F/58	SCLE	3	Hydroxychloriquine (+) Thalidomide (−)

*(+), positive effect of therapy; (−), no effect from therapy; (ci), contra-indicated because of severe side-effects.

The criteria for inclusion in the study comprised: histologically confirmed DLE or SCLE; stable or progressive disease at initial consultation; measurable lesions; no specific treatment for at least 1 month before IFN therapy and no concomitant therapy that could influence the disease. The diagnosis of DLE and SCLE was based on both clinical and histological features. None of the patients had systemic LE as defined by the ARA criteria.

Routine laboratory tests, complement levels, antinuclear antibodies (ANA, double-stranded DNA, Ro, La, Sm, RNP and nucleohistones antibodies), circulating immune complexes (CIC) and serum immunoglobulins were determined at the initial examination and after every 4 weeks. Biopsies of skin lesions were taken before treatment from all patients and processed for routine histology and for direct immunofluorescence. Biopsy specimens were also taken from five patients during and at the end of the treatment with IFN-α.

Interferon α2a (Roferon-A®) administration

Recombinant IFN-α2a (Roferon-A®) was provided by Produits Roche (Paris, France) as lyophilized powder in vials of 3·0, 9·0 and 18·0 × 10^6 U. Patients were treated for 4–13 weeks with doses of IFN-α2a that varied from 18–120 × 10^6 U/week and were adjusted according to the clinical response.

Evaluation of response to therapy

At the initial visit and at 2-week intervals the patients were assessed and indicator lesions monitored for the following symptoms: erythema, infiltration, hyperkeratosis/scaling. If present before treatment, the symptom was given a score of 4, whereas if absent its score was 0. During therapy an improvement of less than 50% scored 3, a major improvement (> 50%)

scored 2, and complete clearance of the lesions scored 1. An exacerbation was recorded as 5. A total score was obtained for each patient at each visit by adding the scores of the indicator lesions. A similar scoring system for the response to IFN was used to assess the skin biopsies. The criteria used were surface hyperkeratosis, thinning of epidermis with vacuolar degeneration of the basal cells and the dermal lymphoid cell infiltrate. The person examining the histological sections was unaware of the clinical course of the disease.

RESULTS

A complete response with total regression of the cutaneous lesions was achieved in five of the patients (three DLE and two SCLE). A partial response of more than 50% occurred in three patients (Table 2). Thus, eight out of 10 patients (80%) had an excellent or good response to treatment. The clearance of lesions was rapid, occurring within 2–6 weeks of treatment. However, the IFN-induced improvement of DLE and SCLE was only temporary, and there was a relapse within 2–14 weeks after stopping treatment (Table 2).

Patients with DLE responded more rapidly and to a greater degree than patients with SCLE. An improvement was noted in five out of six DLE patients after a mean treatment duration of 5·6 weeks (4–8) at a mean dose of 35 (18–45) × 10^6 U/week of IFN-α. Three of the four patients with SCLE had improved with a mean treatment duration of 10 weeks (5–13) and a mean dose of 80 (18–120) × 10^6 U/week. One patient with SCLE had an exacerbation of her cutaneous lesions during IFN therapy and another patient was unchanged after 4 weeks of treatment.

Apart from patient 10, no significant adverse reactions were noted during treatment and subsequent follow-up. However, all the patients had a flu-like syndrome associated with the treatment. This side-effect was dose-dependent and in only one necessitated the reduction in the weekly dose of IFN-α from 63 to 27 × 10^6 U. One patient had asthma-like breathlessness during therapy. Laboratory tests revealed no significant abnormalities during treatment apart from three patients with an increase in their liver enzyme levels.

TABLE 2. Clinical results, follow-up and outcome

Patient	Weekly dosage (× 10^6U)	Duration (weeks)	Clinical result*	Relapse (weeks)
1	42	8	CR	Yes/14†
2	42	4	CR	Yes/2
3	45	8	CR	Yes/4
4	21	4	NC	NA
5	42	5	PR	Yes/2
6	18	4	PR	Yes/6
7	100	13	CR	Yes/12
8	120	12	PR	Yes/4
9	18	5	CR	Yes/6
10	42	4	PD	NA

* CR, complete response; PR, partial response; NC, no change: PD, progressive disease; NA, not applicable.

† Time of occurrence of clinical relapse after withdrawal of IFN.

Before treatment four patients had a low titre of circulating antinuclear antibodies (ANA), which, except in one case, did not vary during therapy. In this patient the ANA titre was 1:64 after 1 month of treatment and rose to 1:4096 at the end of therapy; the titre fell to 1:256 1 month later.

Histology

Prior to treatment, all the skin biopsies showed variable degrees of severity and the histological features of LE. At the end of the study, the biopsies taken from the patients who had improved clinically showed a similar improvement of their histology. The patients with a minimal histological improvement showed either a partial response or in one case an exacerbation of their skin lesions. Generally, the most significant improvement was a reduction in the density of the dermal cellular infiltrate.

DISCUSSION

The present study was designed to determine the potential benefit of interferon α (IFN-α) in the treatment of DLE and SCLE over a short period of time. In five of our patients there was a complete and in three a partial response indicating that recombinant IFN-α2a is effective in the treatment of DLE and to a lesser extent SCLE. The 80% response rate suggests that IFN is as effective in treating DLE and SLE as other treatments such as the antimalarial drugs, thalidomide and topical corticosteroids which all have potential side-effects.

DLE was more responsive than SCLE. The only aggravation of cutaneous lesions seen in this study concerned a patient with SCLE, and we feel that IFN treatment is probably better indicated for DLE than SCLE.

The mechanism of IFN-induced improvement in LE remains uncertain. In addition to antiviral and antiproliferative properties, IFN has many other effects on the various components of the immune system that are relevant to autoimmunity. Effects on B cells appear to depend on the dosage. Low IFN concentrations stimulated and high concentrations inhibited immunoglobulin production.[5,6] Suppressor T lymphocytes have been shown to be inhibited, whereas natural killer (NK)-cell activity may be depressed.[7,8] Recent data suggest that SCLE could result from the pathogenic role of antibodies to Ro antigens expressed on the membranes of keratinocytes, through an antibody-dependent cellular cytotoxicity mechanism.[9] The possibility that IFN may inhibit NK-cell activity could account for the positive response observed in this study. An additional effect of IFN-α is the decrease in production of several lymphokines (including IL-1, IL-2 and IFN-γ) in lupus-prone animal models.[10] It may be that IFN-induced improvement of DLE and SCLE is mediated by an anti-inflammatory mechanism down-regulating lymphokine production.

In all cases in this study there was rapid relapse of the skin lesions after stopping IFN. This relapse is evidence supporting an IFN-mediated improvement of the skin condition in DLE and SCLE and against a non-specific, placebo effect. The fact that improvement was only temporary does not diminish the value of IFN-α as a new approach to the management of DLE and SCLE.

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Plasma exchange in refractory cutaneous vasculitis

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SUMMARY

Eight patients with intractable cutaneous leucocytoclastic vasculitis were given a trial of plasma-exchange therapy. All but one improved, five substantially. Four have been successfully treated by intermittent plasma exchange for periods of 5–12 years. Apart from one episode of hepatitis B, possibly related to administration of fresh frozen plasma, no major adverse effects have occurred. Plasma exchange can be a valuable therapeutic option in patients with severe refractory cutaneous vasculitis.

Severe cutaneous vasculitis can cause substantial morbidity and may not respond to aggressive systemic therapy with oral corticosteroids, dapsone, and various immunosuppressive agents. Since there is at least circumstantial evidence for the role of immune complexes in some patients, the removal of such factors by plasma exchange (PE) appeared a logical development.[1]

Eight patients with intractable leucocytoclastic vasculitis in whom systemic therapy had proved ineffective or had produced intolerable side-effects were referred for a trial of plasma exchange (PE). This report describes their initial clinical response and the long-term outcome.

METHODS

The patients consisted of five females and three males (age range 31–65 years) with severe chronic cutaneous vasculitis (Table 1). Patient 1 has been reported previously.[2] Patients 1 and 2 presented with severe urticarial vasculitis characterized by painful raised erythematous lesions which resolved after 24 h to leave purpuric staining. Patients 4–8 had recurrent papular and purpuric eruptions predominantly confined to the lower limbs, which, in patient 5, had associated skin necrosis with ulceration. Cutaneous manifestations predominated, though arthralgia was also noted at some stage by all the patients. In addition patient 3 exhibited a number of extracutaneous manifestations in association with painful subcutaneous nodules that

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TABLE 1. Clinicopathological features of patients with refractory cutaneous vasculitis

Patient	Sex/Age (years)	Clinical/ pathological features	Direct immunofluorescence	Duration of symptoms before PE (years)	Associated disorders	Abnormal investigations	Response to PE	Current therapy
1	F/31	Urticarial vasculitis	Perivascular immunoglobulin and C_3	16	None	Low C_3, C_4 CIC present	Excellent	Two PEs every 2 weeks (209 in 10 years)
2	F/65	Urticarial vasculitis	—	7	None	ANA weakly positive (DNA binding normal) RhF positive CIC not detected	Good	Cyclophosphamide 75 mg daily (difficult vascular access prevented long-term PE)
3	F/37	Leucocyto-clastic vasculitis	Negative	11	Gut and respiratory tract involvement	CIC present	Excellent	PE 2-weekly as required (134 in 12 years) prednisolone 0–20 mg/day
4	F/55	Necrotizing vasculitis	Perivascular immunoglobulin and C_3	14	Mild bronchiectasis Myeloma with amyloidosis (1988)	IgA paraprotein 4g/l (1981) CIC present	Excellent	PE monthly (74 in 6 years)
5	M/57	Leucocyto-clastic vasculitis	—	3	Pericarditis at onset	ANA positive (speckled) (DNA binding normal) CIC present	Partial	Spontaneous remission, no current therapy
6	F/28	Leucocyto-clastic vasculitis	Negative	3	None	RhF positive CIC present	Partial	Prednisolone 10–15 mg/day
7	M/63	Necrotizing vasculitis	Linear IgM, at dermo–epidermal junction	4	Mild erosive arthropathy	Low C_3, C_4, CH_{50} CIC present	Excellent	Two PEs every 2 weeks (191 in 5 years) Prednisolone 10 mg/day
8	M/50	Leucocyto-clastic vasculitis	Negative	4	Peripheral vascular disease	CIC not detected	Negligible	Azathioprine 200 mg/day

Abbreviations: PE, plasma exchange; ANA, antinuclear antibodies; RhF, rheumatoid factor; CIC, circulating immune complexes by C1q binding and/or rheumatoid factor binding assays.

occasionally became ulcerated. These included: gastrointestinal symptoms with transient radiological abnormalities; respiratory involvement with laryngeal oedema, wheezing and haemoptysis; and, on two occasions, neurological symptoms and signs caused by lesions of the lumbar meninges.

All patients had been treated with numerous drugs, including corticosteroids and one or more cytotoxic agents (azathioprine, melphalan, cyclophosphamide). Some had also received dapsone, isotretinoin, thalidomide, and danazol, without any improvement. Two had tried elimination diets without benefit.

Skin biopsies from all patients revealed a leucocytoclastic vasculitis with fibrinoid change affecting the cutaneous blood vessels. Direct immunofluorescence studies of lesional skin were performed in six cases. Two patients (cases 1 and 4) had granular deposits of immunoglobulin and C3 in a perivascular distribution and one (case 7) had linear deposits of IgM along the dermo-epidermal junction (Table 1).

Patients with an identifiable aetiology or with a significantly elevated level of cryoglobulins (> 100 μg/ml) have been excluded. Complement profile, autoantibodies, rheumatoid factor, immunoglobulins, serum protein electrophoresis, cryoglobulins, anti-neutrophil cytoplasmic antibodies,[3] hepatitis B antibodies (and surface antigen), VDRL and circulating immune complexes by C1q and rheumatoid factor binding assays have been assessed in all the patients. Abnormal laboratory investigations are listed in Table 1.

Plasma exchange was performed with a Haemonetics discontinuous flow cell separator. Vascular access was by antecubital venepuncture, or through a short-term double-lumen central venous catheter, or via a surgically-created subcutaneous arteriovenous fistula for long-term therapy. Initial treatment generally consisted of three 4-l plasma exchanges for 5% human albumin in 1 week. For maintenance therapy, one or two 4-l exchanges were carried out every 2–6 weeks. Because of shortages of blood products we have recently used 1 l of a gelatin plasma expander (Polygeline) together with 5% albumin during maintenance therapy, with no apparent change in response.

RESULTS

Seven of the eight patients improved within 2 weeks of starting PE (Table 1). The five patients who responded well showed a dramatic clinical improvement within the first week, and usually within 12–24 h of their first plasma exchange. Arthralgia and malaise were relieved within 48 h. One patient was unable to continue treatment because of difficulties with vascular access, but the other four have continued to receive plasma exchange as the mainstay of their therapy for periods of 5–12 years. The excellent response to treatment has been maintained, and apart from one episode of hepatitis B possibly related to the administration of fresh frozen plasma, no major adverse effects have been recognized. Three patients did not show a worthwhile benefit from plasma exchange. Two showed only slight improvement within 2 weeks and one failed to respond. Two of these patients are still receiving immunosuppressive therapy.

DISCUSSION

Plasma exchange is widely used in severe systemic disorders where there are known or suspected circulating immunological mediators, such as autoantibodies or immune complexes.[4] There may be a rapid response to PE alone, as in myasthenia gravis or cryoglobulinaemia, but sustained improvement generally depends on the concurrent use of immunosuppressive drugs, as in Goodpasture's disease and systemic vasculitis.[5] As well as the removal of immunoglobulins and immune complexes, PE may exert effects by depletion of complement components and clotting factors involved in tissue injury, by a reduction in blood viscosity, or by improvement in reticuloendothelial system function.[1,4,5]

Plasma exchange has also been used to treat dermatological disorders such as corticosteroid-resistant pemphigus[6,7] and pemphigoid,[8,9] and there are occasional reports of its beneficial effect in pyoderma gangrenosum.[10,11] Of the systemic diseases with major cutaneous manifestations, cryoglobulinaemia often responds well to plasma exchange alone,[4,12] but in most others, including primary systemic vasculitis, the role of PE is to bring about a remission which can then be maintained by drugs.[5,13]

The cutaneous vasculitides represent a heterogeneous group of disorders and in most cases the pathogenesis is unclear. However, the close clinical and pathological similarities between leucocytoclastic vasculitis and the Arthus reaction suggest that tissue deposition of immune complexes may be important in cutaneous vasculitis. Circulating immune complexes have been found in many cases of cutaneous vasculitis, and direct immunofluorescence studies of lesional skin often show deposition of immunoglobulins and complement components in the affected tissue.[14,15] Circulating immune complexes were detected in six of our eight patients, and it is interesting to note that all of these patients showed some response to treatment. None of our patients had antibodies to neutrophil cytoplasmic components; such antibodies have been strongly associated with the major systemic vasculitides (Wegener's granulomatosis, microscopic polyarteritis), though they may not be detected in more limited (single organ) forms of Wegener's.[3]

There have been few reports of the use of PE in isolated cutaneous vasculitis,[1,2] though therapeutic success has been described in Waldenstrom's hypergammaglobulinaemic purpura, which bears a number of similarities to our cases.[16] This may be partly due to the difficulties associated with its use as a maintenance therapy and because it is time-consuming and costly. However, our experience suggests that it can be a highly effective and well-tolerated out-patient therapy in patients with severe cutaneous vasculitis which is unresponsive to conventional therapy.

ACKNOWLEDGMENTS

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Epidermolytic hyperkeratosis: generalized form in children from parents with systematized linear form

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SUMMARY

Two unrelated families are presented in both of which a child with generalized epidermolytic hyperkeratosis (congenital bullous ichthyosiform erythroderma) had a parent with linear epidermolytic hyperkeratosis (epidermolytic epidermal naevus). Light and electron microscopy of skin biopsies of lesions from the children and parents showed typical epidermolytic hyperkeratosis. Gonadal mosaicism in patients with linear epidermolytic hyperkeratosis may be responsible for transmission of the abnormality to the offspring.

Epidermolytic hyperkeratosis (EH) comprises a spectrum of clinical conditions. Three major clinical types appearing at birth or in early childhood have been recognized on the basis of distribution of lesions: a generalized type known as bullous congenital ichthyosiform erythroderma, a linear localized type and a diffuse palmoplantar type. The pattern of inheritance of epidermolytic hyperkeratosis has not been completely clarified. While both generalized and palmoplantar EH can be inherited as autosomal dominant traits[1–4], there is no evidence that linear EH can be transmitted as a linear localized form. However, a few observations have suggested that patients with linear EH can give birth to children with generalized EH.[5–7] We describe two additional families with this pattern of inheritance, adding to the evidence of a genetic relationship between the linear and the generalized form of EH.

CASE REPORTS

Family 1

This 12-year-old girl was born with generalized erythema and with focal superficial erosions of the skin. During the first weeks of life her skin became scaly and then progressively thickened over the years. Physical examination revealed a generalized hyperkeratosis with erythema and areas of blisters (Fig. 1a). Thick verrucous scales with transverse striations were observed in the

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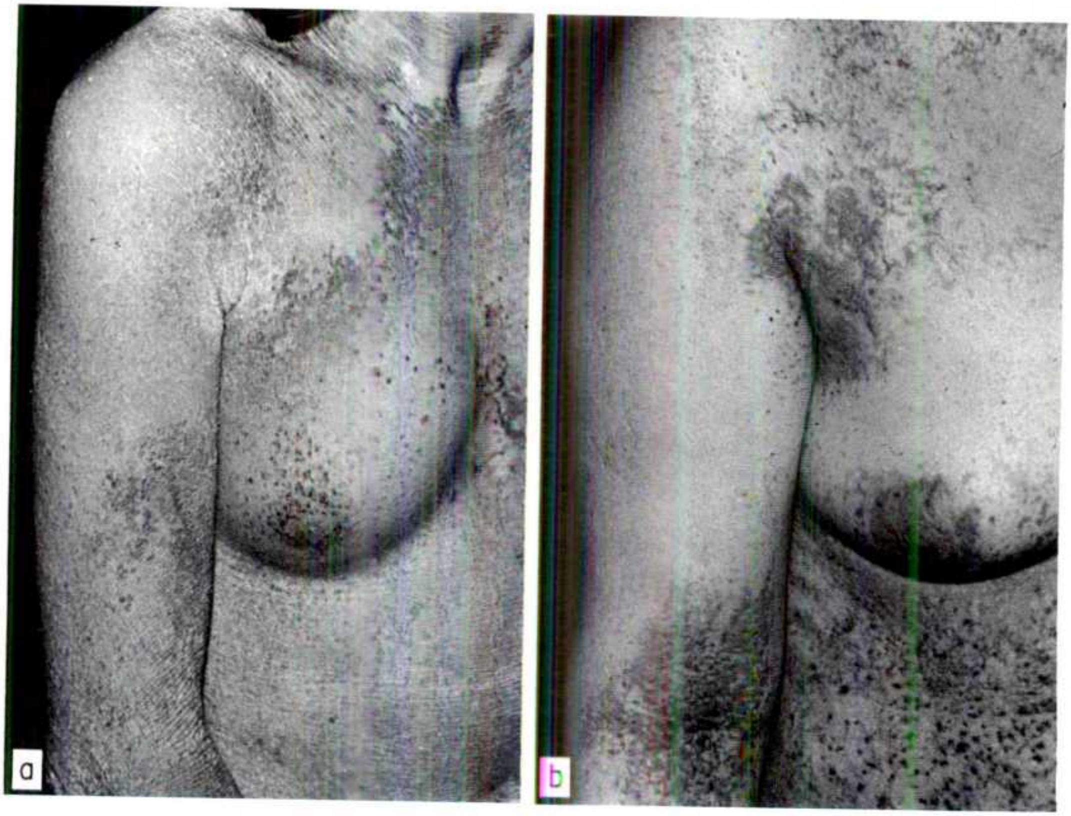

FIGURE 1. (a) Twelve-year-old girl with generalized epidermolytic hyperkeratosis. (b) Streaky and reticulated configurations of verrucous lesions in her mother with areas of normal skin (family 1).

major skin folds. The skin on the face was slightly hyperkeratotic while the palms and soles were normal. Routine laboratory investigations and X-rays of thorax and spine were normal. Light and electron microscopy of the skin showed changes typical of epidermolytic hyperkeratosis with a compact hyperkeratosis and clumps of tonofilaments and cytolysis in the suprabasal keratinocytes. Treatment with oral etretinate 0·5–1 mg/kg per day reduced the hyperkeratotic changes, but treatment was discontinued after a few months because of a marked increase in the skin fragility.

Her mother, aged 43 years, was born with an apparently normal skin and the first verrucous lesions appeared during infancy with progressive extension and thickening. Physical examination revealed a healthy women with bilateral asymmetric linear and reticulate configurations composed of 2–4 mm brown hyperkeratotic papules that followed the lines of Blaschko (Fig. 1b). Most of her body surface was involved but the face, palms, soles and mucous membranes were spared. Her hair and teeth were normal and there were no skeletal, nervous system or other abnormalities. Light and electron microscopic studies on a biopsy of hyperkeratotic skin from the trunk showed the changes of epidermolytic hyperkeratosis (Figs 2 and 3). No abnormalities were observed in the two biopsies obtained from uninvolved skin from the left arm and back.

The girl's sister and father were examined and found to be normal. There was no family history and no consanguity.

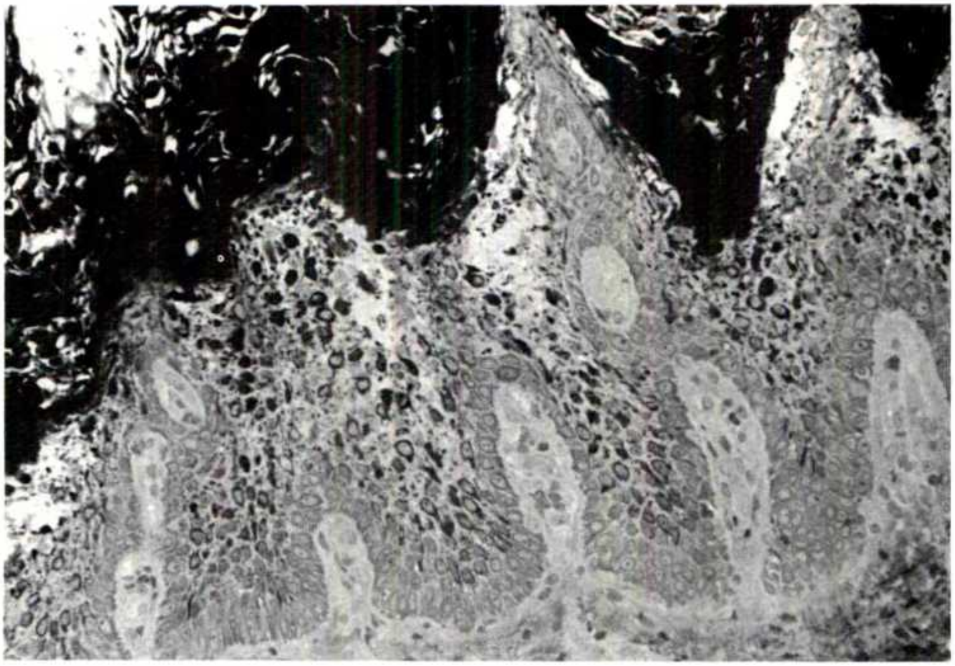

FIGURE 2. Semi-thin section of a hyperkeratotic skin lesion from the trunk of the mother of family 1 showing compact hyperkeratosis with large keratohyalin-like granules and cytolysis in suprabasal keratinocytes (× 100).

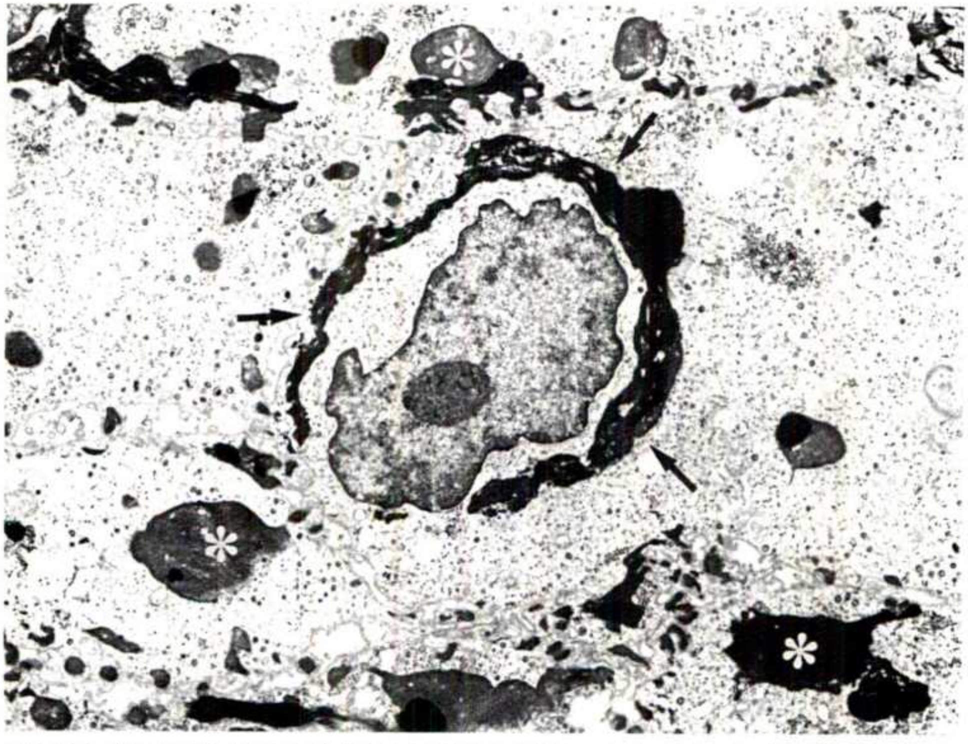

FIGURE 3. Electron micrograph of the same specimen of Figure 2 showing typical tonofibrillar clumps (asterisks) and perinuclear shells (arrows) in suprabasal keratinocytes (× 10 000).

Family 2

A 7-year-old boy had at birth generalized hyperkeratosis. Erythema and bullous lesions appeared a few days later and, over the years, the skin progressively increased in thickness forming transverse streaks in the major skin folds though the blistering became less frequent. Physical examination showed generalized erythema, hyperkeratosis with thick verrucous areas most prominent on the major folds (Fig. 4a) and superficial skin erosions. The face, palms and soles were only slightly hyperkeratotic. Erosions were present on the legs and trunk. His hair, nails and mucosa appeared normal. Routine laboratory tests and X-rays of thorax, spine and long bones were normal. Light and electron microscopy of the skin revealed changes typical of epidermolytic hyperkeratosis. Systemic treatment with etretinate at a dose of 1 mg/kg per day resulted in a marked reduction of the hyperkeratosis without significant increase in skin fragility.

His father, aged 40 years, had normal skin at birth and at the age of 3 months warty streaks appeared and gradually spread to involve most of the body. He appeared healthy with bilaterally asymmetric verrucous lesions disposed in streaks and whorls along Blaschko lines, especially on his flanks (Fig. 4b). The genital area was also involved but the face, palms, soles and mucous membrane were spared. His hair and teeth were normal and there were no skeletal or nervous system anomalies. Typical changes of epidermolytic hyperkeratosis were seen on light and electron microscopy of a skin biopsy from a hyperkeratotic lesion on the right flank. A biopsy taken from clinically uninvolved skin from the same flank was normal.

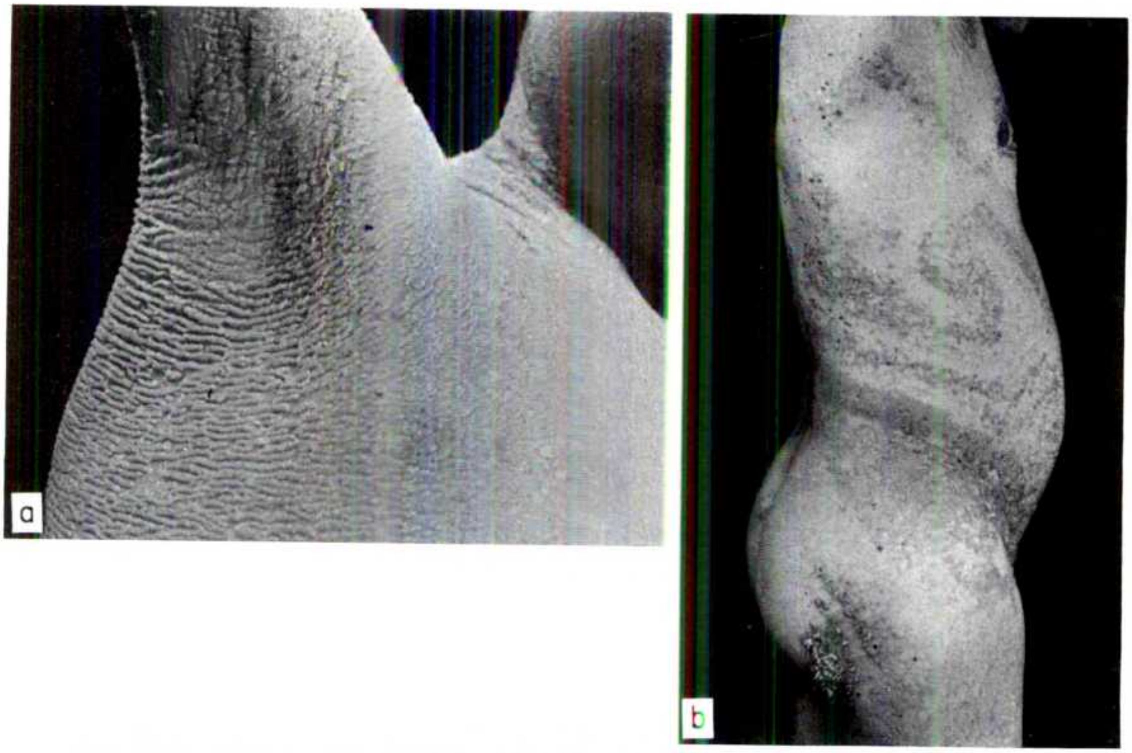

FIGURE 4. (a) Thick verrucous scales with transversal striations on the axillary folds in a 10-year-old boy with generalized epidermolytic hyperkeratosis. (b) Flank showing the striking pattern of verrucous lesions in streaks conforming to Blaschko lines in his father (family 2).

The boy's brother and mother were examined and found to be normal. There was no history of other affected members and no consanguinity.

DISCUSSION

Epidermolytic hyperkeratosis (EH) is a tonofilament system disorder ultrastructurally characterized by tonofibrillar clumping in the suprabasal layers of the epidermis.[8,9] Histological examination shows a hypergranulosis with compact hyperkeratosis and cytolysis of the epidermal cells of the upper malpighian and granular layers.[10] Three major clinical types sharing the same ultrastructural abnormality can be recognized on the basis of distribution of lesions and pattern of inheritance: a generalized type, a linear localized type and a diffuse palmoplantar type.

Generalized epidermolytic hyperkeratosis (GEH), also known as bullous congenital ichthyosiform erythroderma, is a universal skin defect characterized clinically by generalized erythema, verrucous hyperkeratosis and superficial bullous lesions. GEH is inherited as an autosomal dominant trait, although many of the cases are sporadic.[1,2] This disorder is expressed in foetal life as early as 18 weeks and can be prenatally diagnosed.[11,12]

Linear epidermolytic hyperkeratosis (LEH) displays a mosaic pattern of skin involvement with streaky configurations following Blaschko's lines. It appears gradually after birth and presents as a verrucous naevus ranging in extent from a single linear naevus to a unilateral (naevus unius lateris) and bilateral systematized naevus that involves most parts of the body. Widespread LEH could be misdiagnosed as GEH, although this can be clarified by the linear configuration, presence of areas of normal skin and absence of transverse verrucous streaks on the major folds. Widespread LEH should also be differentiated from ichthyosis hystrix (Curth-Macklin) which presents similar clinical and histologic features. This rare type of ichthyosis can be identified by electron microscopy that reveals aggregation of tonofilaments in concentric perinuclear shells with no clump formation.[13] To our knowledge LEH has never been reported to be transmitted as a localized form, but in three reports it has been observed in parents of children affected with GEH.[5-7]

Palmoplantar epidermolytic hyperkeratosis (PPEH), also known as Vörner palmoplantar keratoderma, does not display a mosaic pattern of skin involvement. It is probably the most common inherited diffuse palmoplantar keratoderma[3] and presents the same clinical features as Thost-Unna disease. More than 30 family reports have established an autosomal dominant mode of inheritance[3,4] and, to date, there is no evidence of any linkage with GEH.

EH may even occur in acquired skin conditions such as epidermolytic acanthoma[14] and, as an incidental histological finding, in a variety of other acquired solitary skin lesions.[10]

Our two families have the same pattern of inheritance of epidermolytic hyperkeratosis, confirming a link between LEH and GEH. It has been suggested that the localized form might be a mosaic arising from a mutational event occurring during early embryogenesis.[15,16] This hypothesis could provide an explanation for many of the puzzling aspects of LEH: the distribution of skin lesions along the Blaschko's lines, the wide range of phenotypes, the evidence that this disorder is not transmitted as a localized form and the possibility of GEH in the offspring. The gene mutation could involve all of the organs including the gonads, and if the children of such a mosaic patient carry the mutation they will have it in all of their cells thus exhibiting the generalized form of the disease.

Careful examination of both parents of a child with GEH is important in order to provide genetic counselling to the family. The risk of recurrence of GEH in future offspring is 50% if one parent is affected with GEH. The risk for a parent affected with LEH is difficult to estimate. According to the mosaic hypothesis the risk depends on the percentage of gonadal cells which are involved by the gene abnormality and should range from 50% if all the cells were involved to 0% if no cells were involved. A correlation between the extent of cutaneous involvement and the likelihood of gonadal mosaicism could also exist. Both our patients with LEH had widespread, systematized lesions and could carry a higher risk than patients with small localized LEH. This pattern of inheritance may not be rare and it is possible that some cases of GEH reported as sporadic actually had a parent with LEH which remained unnoticed.

Our observations also emphasize the importance of systematic histological examination of all cases of linear epidermal naevus to look for epidermolytic hyperkeratosis. Patients with LEH should be informed about the risk of transmission of epidermolytic hyperkeratosis with a diffuse skin involvement to the next generation and about the possibility of prenatal diagnosis of GEH in the offspring by ultrastructural examination of foetal skin biopsies.

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PEDIATRIC DERMATOLOGY

Editors

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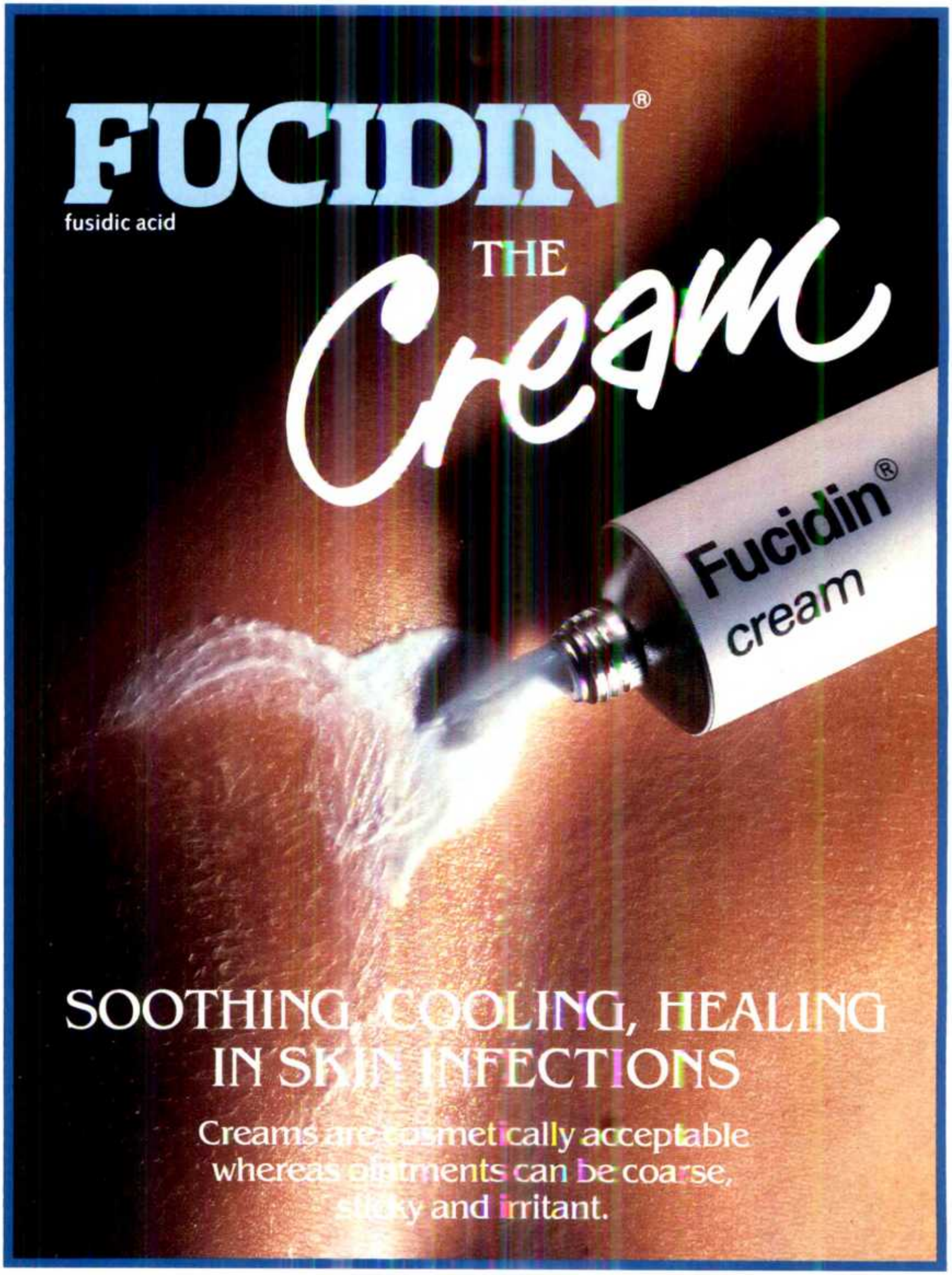
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Erythropoietic protoporphyria and iron therapy

SIR, With reference to the report by Milligan *et al.*[1] of four cases of erythropoietic protoporphyria (EPP) which appeared to be exacerbated by oral iron therapy, we wish to report a case of EPP whose response to oral iron has varied on different occasions. The patient was not previously known to have EPP and in the past had taken oral iron without untoward effects but presented with a florid exacerbation of EPP 4 days after starting ferrous fumarate and discontinuing an oral contraceptive preparation.

The 27-year-old female patient complained of headache and the only abnormal finding was a microcytic anaemia (haemoglobin 9·6 g/dl (normal 12–18), mean corpuscular volume 67 fl (normal 80–99) and a low serum ferritin 16 μg/l (normal 40–70)). Ferrous fumarate 580 mg daily was commenced and the contraceptive pill Norimin (Norethisterone 1 mg plus ethinyloestradiol 35 μg) which she had taken for the previous 3 months was discontinued. She had never been on oral iron and the contraceptive pill at or around the same time. Four days later she developed an intense burning sensation of the skin on the dorsum of both hands that was relieved only by immersing the hands in cold water. She had, to a lesser degree, burning of the skin on the face. Retrospectively, she recalled several similar but less severe episodes of rash that were associated with exposure to sunlight. However, this episode occurred during dull weather and was the worst she had ever experienced. On examination there was gross oedema of both hands with an erythematous and purpuric rash over the metacarpal heads induced by rubbing and there were a few pitted scars on the bridge of the nose.

The red blood cell fluorescence was strongly positive and the red cell protoporphyrin level was elevated at 21 004 nmol/l (normal 70–780 using Heller method) 5 days after the last dose of oral iron when the exacerbation was at its peak and fell to 12 600 nmol/l 9 days later. The liver enzymes were initially elevated with gammaglutamyl transferase 65 μ/l (7–46), aspartate transaminase 76 μ/l (10–40) and alanine transaminase 194 μ/l (10–45), but these subsequently became normal. Her alcohol intake was 10 units per week. An ultrasound scan of her abdomen showed no evidence of gallstones and no other abnormality and a percutaneous liver biopsy revealed normal histology.

This patient has responded differently to oral iron at different times and this is not consistent with the theory of Milligan *et al.*[1] that there are two distinct subgroups of erythropoietic protoporphyria distinguished by their response to iron. Previously it was believed to be important to correct iron deficiency in EPP[2] as this caused an increase in protoporphyrin production. Avner *et al.*[3] noted that raised protoporphyrin levels in an animal model induced cholestasis. The oral contraceptive pill can also induce cholestasis[4] which can reduce the excretion of protoporphyrin in bile. The liver enzyme profile in our patient would have been consistent with a degree of cholestasis, perhaps related to the oral contraceptive therapy or protoporphyria itself and may have modified the response to oral iron on this occasion. Milligan *et al.*[1] do not state the dose of iron prescribed for their patients but our patient received a relatively large dose of 200 mg of elemental iron daily. It may be that the state of the iron stores when oral iron is administered, the dose and preparation of iron prescribed or concomitant medication can influence the response to oral iron in EPP.

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Lichen planus following HBV vaccination: a coincidence?

SIR, A 30-year-old woman who was at risk from hepatitis B virus infection (HBV) and whose father had a post-viral chronic active hepatitis (CAH), received HBV vaccine HEVAC B (Pasteur Institute) and had three 1-ml doses of the vaccine in April, May and June 1987. In early July, she developed a number of slightly itchy, small, erythematous papules on the ankles, feet, hands, fingers and forearms. The lesions gradually increased in number and size. By October, there were a number of typical small lichen planus (LP)-like papules, and the diagnosis of LP was confirmed on histology and with direct immunofluorescence. A polyclonal antibody to the most frequent HBsAg epitopes (Behring, Marburg, F.R.G.) was tested on the lesional skin using an immunoenzymatic assay, but this gave negative results. The eruption was treated with a corticosteroid cream and cleared within 1 month.

Routine laboratory investigations, including the liver enzymes and bilirubin were all normal. In September 1987, she had a low titre of anti-HBs antibodies (16 mIU/ml), but in July 1988 both anti-HBs and anti-HBc antibodies were negative.

The occurrence of LP in an HBV-vaccinated patient may be a coincidence, but may be causally related. The association of LP with chronic hepatitis has been reported many times in recent years and is not infrequently seen in primary biliary cirrhosis,[1] though in Italy CAH has been more frequently diagnosed.[2,3] The prevalence of CAH patients with LP is much higher than expected and this was also found in the U.S.A.[4] The discrepancy between the findings in Italy and in the U.K. have been attributed to a higher prevalence of HBV infection in Italy.[5,6]

Our patient received a plasma-derived HBV vaccine that originated from native HBsAg particles including the S and pre-S proteins. In the HBV genome the two genes, S and pre-S, code for the proteins of the viral envelope and the pre-S gene includes two regions (pre-S1 and pre-S2) coding for two specific proteins. Native HBsAg is a mosaic of epitopes (S, pre-S1 and pre-S2), each of which induce specific antibodies. Our patient may have been sensitized to one of these proteins, and it is possible that the lichen planus occurred as a graft-versus-host reaction to keratinocytes expressing one of the sensitizing proteins or a similar epitope. We would be interested to know how many HBV vaccinated individuals develop lichen planus and how they respond with respect to seroconversion.

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Cyclosporin A in psoriasis: interaction with carbamazepine

SIR, We report a man who received high doses of cyclosporin A for psoriasis, with no clinical improvement and low blood levels of cyclosporin A, until concurrent anti-epileptic treatment with carbamazepine was changed to sodium valproate. We believe this is due to the induction by carbamazepine of the hepatic cytochrome P450 enzyme system which is the major route of elimination of cyclosporin A.

A 44-year-old man with an 18-year history of severe erythrodermic and sometimes pustular psoriasis had received systemic treatment continuously since 1981. His drugs included methotrexate, razoxane, etretinate and chlorambucil. He was admitted with a further episode of erythroderma in October 1986. He had a past history of alcohol abuse and a liver biopsy in 1981 showed a minor degree of fatty change. Repeated annual liver ultrasounds had shown mild hepatomegaly but his liver function tests had been normal for 3 years. He had suffered from suspected epilepsy since 1971, with a normal electroencephalogram and CT brain scan. He had had no fits since 1981 while on carbamazepine 200 mg b.d. On admission he was erythrodermic and all his systemic treatment (excepting carbamazepine) was stopped, namely methotrexate, etretinate and chlorambucil. Four days later he was commenced on cyclosporin A at a dose of 3 mg/kg per day which was gradually increased to 20 mg/kg per day (1200 mg/day). Despite this high dose his cyclosporin A whole blood levels, monitored weekly as a 24-h trough level by radioimmunoassay using polyclonal antiserum, remained subtherapeutic and he showed no clinical improvement. Carbamazepine was changed to sodium valproate and within days his cyclosporin A blood level rose and his psoriasis improved (Fig. 1). His renal function remained normal throughout as measured by weekly creatinine clearance.

Interactions between cyclosporin A and other drugs are well recognized. Cyclosporin A has been shown to be of benefit in the treatment of severe psoriasis,[1,2] but it has a low therapeutic index and an understanding of its pharmacokinetics is helpful.[3] The major route of elimination of cyclosporin A and its metabolites is via biliary excretion, urinary excretion accounting only for about 6%. The cytochrome P450 system, which is made up of at least eight isoenzymes in man, is thought to be responsible for cyclosporin A metabolism. Drugs believed to inhibit the cytochrome P450 system, eg. ketoconazole and erythromycin,[4] have been reported to decrease cyclosporin A elimination. In contrast, drugs which induce this enzyme system appear to accelerate cyclosporin A metabolism; reported examples are rifampicin and phenytoin and one case of carbamazepine.[5] We report this as the second case, to our knowledge, of reduction in cyclosporin A blood levels apparently due to induction of hepatic cytochrome P450 by carbamazepine. The ability to obtain therapeutic blood levels of cyclosporin A by substitution of concurrently

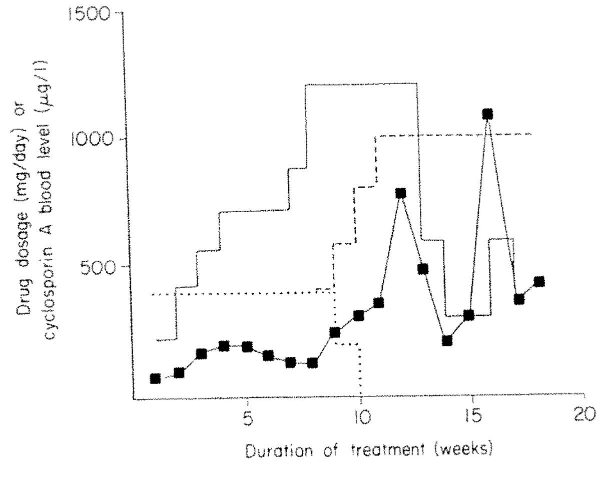

FIGURE 1. Changes in the cyclosporin A blood levels (—■—) and in the doses of cyclosporin A (——) carbamazepine (······) and sodium valproate (––––).

administered carbamazepine with sodium valproate highlights an alternative method of treatment for other epileptic patients who may encounter similar therapeutic problems.

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A controlled-trial of house dust mite eradication using natamycin in homes of patients with atopic dermatitis

Sir, I read with interest the article by Colloff *et al.* (*Br J Dermatol* 1989; **121:** 199–208), and I think it is unfortunate that in a non-selective group it should be stated that there was no clinical benefit from natamycin or house dust mite regimens. We have ignored for too long the important part played by the house dust mite in the aetiology and natural history of atopic eczema. In uncontrolled studies with nocturnal flare atopic children, I have been able to show up to 50% reduction in topical steroid consumption. I accept their comments that uncontrolled studies might be due to the intervention involved, but would urge that in those children with nocturnal flares, eradication of house dust mite is important.

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Book Reviews

A Colour Atlas of Dermato-Immunohistocytology. Edited by HIROAKI UEKI AND HIDEO YAOITA (1989). London, U.K.: Wolfe Medical Publications. Illustrations: 226 colour, 176 black and white. ISBN 0 7234 15846. Price £60·00.

The study of the immunopathology of the skin is becoming an increasingly complex and fascinating field. Developments and improvements in the techniques of investigation are advancing our knowledge of this subject at an ever increasing rate. It is thus useful to have an overview illustrating the current 'state of the art' of technical procedures available to us in cutaneous immunohistology edited by two outstanding investigators in the field.

This book is divided into major sections dealing with technical procedures in immunohistology, the use of these procedures is enhancing our understanding of the components of normal skin and the findings in various dermatological diseases states. Each section covers a comprehensive range of topics with a page of text on each topic and one to several pages of illustrations. The major section is the immunopathological findings in various dermatoses. Each item includes a brief discussion of the pathogenesis, clinical findings and pathology of the disease with a more detailed study of immunopathology. There is a particular emphasis on practical points such as the ideal site of biopsy and handling and fixation of the specimen.

As the format is that of an atlas, the explanatory text is necessarily very brief. The procedures illustrated and discussed include immunofluorescence, immunohistochemistry in general and immunoelectron microscopy. There is also particular reference to the application of monoclonal antibodies in dermatopathology. The practical points are useful but it is unfortunate that the general readability of the text is hampered by poor translation in parts.

Most of the illustrations and diagrams are in full colour and they are excellent in both quality and lay out, making this a very attractive book. The scanning immunoelectronmicroscopy and three-dimensional immunofluorescence micrographs were particularly spectacular. The tables are well thought out and easy to interpret.

Despite the fact that any book about such a rapidly changing field is destined to become out-dated soon after publication, this atlas should prove to be a useful and attractive addition to the libraries of dermatologists and pathologists with an interest in the immunopathology of the skin. It would be particularly useful in giving a general summary on the use of immunohistocytological techniques in fields other than the individual's particular area of expertise.

M.M.BLACK
E.M.WILLSTEED

Opportunistic Infections in Patients with the Acquired Immunodeficiency Syndrome. Infectious Disease and Therapy Series No. 3. Volume Editors: G.LEOUNG AND J.MILLS. (1989). Series Editors: B.E.SCULLY AND H.C.NEU. New York: Marcel Dekker. Pp. 476. ISBN: 0-8247-8080-9.

Infection with HIV, the causative agent of AIDS and the AIDS-related syndromes, ultimately leads to the progressive destruction of the sufferer's immune system, inevitably rendering the patient susceptible to an ever-expanding list of exotic and previously unrecognized conditions, many of which may be life-threatening. Somewhat perversely, the emergence of AIDS in the 1980s has lead to major advances in knowledge concerning two broad aspects of infection. Firstly, much new information concerning the whole field of opportunistic infection (OI) has emerged since the ability of many previously unrecognized pathogens to infect immunocompromised human beings in certain circumstances has become clear. Secondly, the existence of a pool of patients with HIV-related disease suffering from these opportunistic infections has lead to the development of more effective and specifically targeted chemotherapeutic agents for controlling previously almost untreatable viral, fungal and protozoal infections.

In the main, this compact volume serves a valuable role by sensibly concentrating the bulk of its attention on this enormous and expanding topic of HIV-related OIs. Indeed, it might well have been improved by the editors restricting themselves more closely to matters more directly relevant to the book's title. For example, the reader is initially subjected to a somewhat irrelevant epidemiological overview of the HIV/AIDS situation (which perhaps concentrates too much on the USA to be of much use to the reader working outside that country) and similar criticisms might be levelled at the section dealing with the immunology of HIV infection; both these sections are far too slight to be of much value to any serious student of HIV epidemiology or immunology and should perhaps have been omitted to allow more space to be devoted to the area in which the book is most likely to be of greatest value, namely in acting as a comprehensive and up-to-date reference text for HIV-associated OIs. This is a formidable enough task in itself; for example, an enormous amount of new data was first revealed to the world at the 5th World AIDS Conference in Montreal in June 1989 and it will be the same at the 6th and 7th conferences in San Francisco in 1990 and in Florence in 1991. Nonetheless, apart from these relatively minor criticisms, the book has many strengths. It is well-referenced. The chapters examining specifically neurological and specifically paediatric HIV disease, cutaneous infections, oral manifestations, and opportunistic viral, bacterial, mycobacterial, fungal and protozoal diseases deal very fully with the range of potential infecting organisms which might be encountered and the present 'state of the art' with respect to possible therapeutic approaches. It performs sterling service in painting a clear picture of acute HIV infection ('HIV mononucleosis') which affects about 25% of HIV-infected individuals and may well be the disease's first presenting feature, and also provides a useful appraisal of the specific health problems (often dermatological) which might be encountered in HIV-infected patients emerging from Africa. Furthermore, other chapters give helpful hints concerning the clinical assessment of HIV-infected patients who might be suffering from an OI, taking into account relevent history taking, clinical examination and appropriate laboratory tests. Another helpful section looks at specific clinical scenarios such 'fever' and 'diarrhoea' and underlines the enormous differential diagnosis which might exist in such situations.

The fundamental problem with books such as this one remains regrettably that they rapidly become out of date. Even now this is the case here; for example, the book contains no mention of work incriminating *Microspora* spp. as diarrhoea-inducing agents in AIDS, or *Actinobacillus* spp., black pigmented *Bacteroides* and *Wolinella* spp. as the possible causative agents in HIV-related periodontitis. Obviously, therefore, this book will require regular revision if it is to remain both comprehensive and abreast of developments, and this may well render it an expensive asset if successive editions are to be purchased as they appear in print. However, as things stand in the latter half of 1989, this book is an up-to-date and admirably comprehensive provider of information about most of the OIs recognized to occur in HIV sufferers and the therapeutic approaches available to treat them, and should be of particular value to physicians and microbiologists who are regularly responsible for the care of such patients, although it should be remembered that all doctors may come across HIV-infected patients in their everyday work.

S.I.GREEN

Acne. WILLIAM J.CUNLIFFE (1989). London: Martin Dunitz. Pp. 391. Illustrations: innumerable. ISBN 0-948269-39-1. Price £65·00.

A bumper sticker, attributed to Woody Allen, states 'Life is a bitch—and then you die' perhaps could be rewritten as 'Adolescence is a bitch—and then you get acne'. The subject is the butt of many jokes and considerable misery to many teenagers and young adults. Many myths exist concerning the causation and treatment of this condition. Dr Cunliffe and his group in Leeds are well respected for the clear, logical, scientific research they have undertaken in this subject. This book is a complete text on the subject and the contents reflect the knowledge and enthusiasm of the author.

The 20 chapters can be loosely divided into three groups, the first six chapters concern the clinical aspects of the disease, the next nine chapters covers scientific research on acne, and the final five chapters describe the various therapeutic modalities.

The clinical section includes 66 pages describing the clinical features of acne with a very solid description of the evolution of the various lesions of acne. It is probably worth mentioning at this point that the quality of the clinical photographs and their reproduction in the book are excellent. This makes the subtle clinical features mentioned in the text quite understandable. A section on non-acne disorders of the pilosebaceous unit includes a comprehensive review of hidradenitis suppuritiva. The Leeds technique of grading acne is well described, but it would probably need personal teaching to make one's own attempts reproducible.

Nine very thorough chapters on the physiology, biochemistry, microbiology, inflammatory mediators and animal models of acne then follow. The practising dermatologist will find these fascinating to dip into and gain a much better understanding of the pathophysiology and rationale of treatment of this most common disease. The amount of research in this subject is vast and has been very well distilled by the author; he even allows a little space for those who disagree with his theories. The descriptions of the laboratory techniques are very thorough and in places could be a laboratory manual. The chapter on the microbiology of acne is written by Dr K.T.Holland who gives a concise review of the pathogens found on the human skin and acne involved areas, demonstrating further the complexity of acne.

For the reader who may find the thorough way that Dr Cunliffe has dealt with the research behind the subject too daunting a read, he has produced a 2-page summary which encapsulates the current thinking on the pathogenesis of acne.

The treatment of acne has evolved through serendipity and logical scientific investigation. Dr Cunliffe is honest enough to admit that, in early days, his group may have been responsible for advocating low-dose antibiotic regimens. His group's experience of treating all grades of acne is evident in this section. The treatment section covers topical treatments, antibiotics, retinoids and treatments of side-effects and the sequelae of severe acne.

This book must rank as the definitive work on acne. The text is thorough but always clear. The book is well illustrated with colour plates and clear diagrams. It is perhaps churlish of me to find fault; however, there was a 1000-fold inaccuracy in the dose of ethinyloestradiol. Personally, I prefer references at the end of each chapter rather than at the end of the book. All practising dermatologists should have access to this book. In dermatological centres with an investigational leaning the scientific staff would find it useful. The book would prove of use to the interested paediatrician and endocrinologist and would not be out of place in a large general practice health centre.

S.I.White

Immunological Diseases Volumes 1 and 2 (4th Edition). Edited by M.Samter, D.W.Talmage, M.M.Frank, K.F.Austin and H.N.Clayman (1988). Boston, Toronto: Little Brown. Pp. 2044. Price £145.00.

This is the fourth edition of one of the standard textbooks of clinical immunology and many sections have been extensively revised and updated since the last edition. Perhaps a sign of the times is the interesting addition of a chapter on the immunology of old age.

The book in arranged in major sections composed of basic immunology, non-atopic immunological disorders, atopic diseases, allergic reaction patterns of the skin and finally diseases with immunological features. As the senior editor suggests in the preface, this is perhaps the last edition that will be organized according to disease patterns. In future editions greater emphasis will be on why the majority of the population appears to be protected from or 'immune' to immunological disease. There have been many very significant developments since the last edition, particularly those in molecular biology. The transition from classical basic immunology to molecular immunology has been achieved well in this edition.

The book has multiple acknowledged expert contributors and, in spite of its size, it has been thoughtfully edited so that there is neither repetition nor any significant omission. Many of the chapters are introduced with a summary of the relevant historical events with the main text concentrating on the current state of knowledge. The chapters are often concluded with predictions and possible future developments. On the whole this layout works well and has produced a comprehensive, up-to-date and readable book. It is well referenced with excellent clear line drawings and adequate photographs. The book is to be recommended to all those in medical training requiring an introduction to the ways in which the immune system may prevent or cause disease.

R.J.Pye

Psoriasis and Eczema (Clinical Handbook Series). Edited by L.FRY (1989). Clinical Press. Pp. 202. ISBN 1-85457-002-1. £20.00 (estimated).

This handbook is intended particularly for young doctors working in hospitals and primary care and for those involved in training them. The series has been designed to cover growing points in medicine and the authors have been chosen because they are working both in hospital and the community.

In the preface it states that the co-authors are all from the Dermatology Unit at St Mary's; there is, however, no list of contributors with their appointments, which might make the non-dermatologist reader question the authority of the opinions expressed. There are six chapters with references and an eight page index. Dr Lionel Fry covers clinical features in a concise review illustrated with eleven excellent coloured photographs. Dr C.E.N.Griffiths gives a comprehensive review of the various treatment options. He states in the section of corticosteroids that to achieve quicker alleviation of infiltrated plaques, salicylic acid may be added to the cream or ointment to a concentration of 10%. Many dermatologists would be concerned of the dangers of salicyism at this concentration. Dr B.S.Baker, who is a scientist and not a clinician, follows with an excellent chapter on the aetiopathogenesis of psoriasis based on her work with Professor Helgi Valdimarsson. All dermatologists would benefit from this up-to-date review of recent developments and advances in this field. There is a chapter on the clinical features of eczema by the late Dr R.J.Clayton which covers the various types followed by a chapter on the management of eczema by Dr J.N.Leonard and a chapter on the aetiology of eczema by Dr A.V.Powles. The treatments recommended are both sensible and non-controversial. It is unfortunate that gentian violet paint is recommended for intertrigo as this is no longer readily available because of its carcinogenicity in animals. I could find no section on industrial dermatitis (eczema) which has important medico-legal connotations in the community and any future edition should cover this important subject.

Overall, the book is good value for money and will enhance the knowledge of those training in dermatology and will be a helpful guide to those working in hospitals and the community who wish an up-to-date guide of psoriasis and eczema.

N.MORLEY

Systemic Sclerosis: Scleroderma. Edited by M.I.V.JAYSON AND CAROL M.BLACK (1988). Chichester: John Wiley. Pp. 350. £75.00.

This excellent monograph amply fulfils the editors' stated objectives, to excite interest in scleroderma and to stimulate integrated clinical and basic research. The contributors are all acknowledged experts and there is generally good editorial control with little repetition in spite of the number of authors.

The opening chapters are clear dissertations on the classification, epidemiology and basic underlying pathology of scleroderma. Whilst there are data to suggest a 'susceptibility gene' or even a progression marker for scleroderma, it is still imprecise and it will be interesting to see full restriction-fragment-length polymorphism analysis of MHC, immunoglobulin and T-cell genes. Will it ever be possible to define an immune genotype that will develop scleroderma if exposed to certain environmental agents?

Scleroderma is characterized by the over production of collagen and there is a good clear review of the structure and the metabolism of collagens with sections on the possible roles of proteoglycans and glycoproteins.

Animal models may be invaluable in the study of the early stages of a disease. Review of the current models shows that the tight-skinned (TSK) mouse demonstrates striking similarities to systemic sclerosis, although the animals lack the immunological changes. Further study of the abnormal regulation of collagen in TSK mice may yield important information about scleroderma.

The clinical chapters cover all the relevant sections including endocrinopathies and are generally very thorough. In the section on the lung, the authors have highlighted those references of special interest or importance. In a book so extensively referenced this would have been a valuable feature in other chapters.

The sections on treatment were perhaps the most disappointing, but this may reflect largely on the frustration felt through lack of adequate available treatment. One of the editors has produced a balanced and well referenced section on the treatment of systemic sclerosis, but a number of the treatments suggested in other sections of the book, whilst interesting, are unsubstantiated. Somewhat illogically, a comprehensive table of controlled-treatment trials is to be found in the epidemiology section.

The line drawings are excellent but the clinical photographs are barely adequate for a book costing £75.00.

The book can be thoroughly recommended to both the clinician wanting a comprehensive review of scleroderma and the investigator enquiring about potential growth areas in the subject.

R.J.PYE

A Colour Atlas of Nursing Procedures in Skin Disorders. L.A.STONE, E.M.LINDFIELD AND S.ROBERTSON (1989). London: Wolfe Medical Publications. Pp. 80. ISBN 0-7234-09129. Price £14.95.

This text was compiled to assist general, community and occupational health nurses, health visitors, general practitioners and chiropodists. A number of facets are discussed, including the control of skin disorders by the correct administration of prescribed therapy; education of patients, their families and the general public on skin disorders and healthy skin; and the nurse's role as co-ordinator between patient, their family and skin-care team, and as provider of psychological support.

The Atlas is divided into 44 sections, each covering a different practical aspect. Sections include such diverse aspects as the principles of topical therapy and its correct application, dressing technique in almost every potential variation and need for the same, wart treatment, management of infestation, patch testing, and photochemotherapy. All sections are exceptionally well illustrated, with clarity of description. The section on dressings for ulcers and ulcerated skin, whilst brief, is succinct. This problem is approached by the authors in a manner which illustrates their expertise in the field of dermatological nursing. They describe Turner's criteria for the optimum wound-healing environment and incorporate this into an assessment of the individual patient's needs. Any patient whose nurse reads and learns from this section has been assisted, in absentia, by the authors.

The excellent description on patch testing should also assist nurses in the general field to appreciate the finer points in the nursing management of patients undergoing this procedure.

Whilst in the specialist units throughout the country local idiosyncracies may exist, the only major item in the Atlas identifiable as unfamiliar is the practice of using wet lint as a method of rehydrating dry, flaking eczematous skin. The authors do state that this is only applicable to a very small group of patients. They identify this as only for use under medical supervision in hospital. All procedures which they identify as such are clearly marked in bold print.

In the section on care of the erythrodermic patient, the authors have not resisted the obvious temptation to extend beyond the title of the book: in an atlas of procedures, the nursing management of the erythrodermic patient is perhaps outside its scope. It would have been appropriate in this instance to delete the repetition of the equipment list and application of topical therapy and utilise a problem-solving approach fully. However, the authors do again indicate the need for specialist management and the general nurse has from this an insight into the formulation of a plan of care.

For the nurse outside a dermatology unit, this text should prove to be an invaluable source of reference and learning, particularly in the clear and concise method of presentation.

It should be readily available in nurse education centres where the learner nurse has been in great need of a modern illustrated guide. It should be included in every dermatology sister's library as a teaching tool: the dust would certainly not settle on its covers. The video presentation is awaited. . . .

D.N.BOLAND

Pharmacology and the Skin. Vol. 3, *Pharmacology of Retinoids in the Skin.* Edited by U.Reichert and B.Shroot (1989). Karger: Basel. ISBN 3-80554909-1. Pp. 282. Price £99.00.

While the therapeutic use of retinoids is becoming increasingly popular in dermatology, and we strive to develop new effective derivatives of retinoic acid, the exact mode of action of retinoids is still poorly understood. This excellent book brings together the many papers presented at the 8th CIRD Symposium on Advances in Skin Pharmacology which was held in Cannes in September, 1988.

The book is divided into five sections, the first of which covers the cellular biology of retinoids, and which includes a number of papers on the effect of retinoids on keratinocyte differentiation and modulation of tumour growth. The following sections cover animal pharmacology, pharmacokinetics and safety aspects, clinical applications and, finally, chemical synthesis and future aspects.

As the articles in this book form the proceedings from the CIRD Symposium, the majority are likely to appear as full papers in the future, however the book brings together many excellent reports, and although the price of £99.00 may deter individual purchasers, the book will undoubtedly prove to be an invaluable asset in departmental libraries.

M.Edward

British Journal of Dermatology (1990) **122,** 433.

News and Notices

6th International Congress of Pediatric Dermatology
Toronto, Canada, June 8–11 1992

The 6th International Congress of Pediatric Dermatology will be held in Toronto, Canada, 8–11 June 1992. For further information contact James E. Rasmussen MD, Department of Dermatology, University of Michigan, 1910 Taubman Center 0314, Ann Arbor, MI 48109-0314, U.S.A. Telephone: (313) 936-4086, FAX (313) 936-6395.

3rd International Skin Therapy Symposium, Brussels, 17–19 May 1990

The 3rd International Skin Therapy Symposium will be held in Brussels at the International Conference Centre (Heyzel), Brussels, from 17 to 19 May 1990. There is an international faculty of speakers. For further information contact Dr Geert Cauwenbergh, Janssen Research Foundation, Turnhoutseweg 30, B-2340 Beerse, Belgium.

The Psoriasis Association
Josie Bradbury Travel Award, 1990

Applications are invited for the 1990 Josie Bradbury Travel Award, the amount of the award being £800, from those interested in the study of any aspect of psoriasis and its treatment. The forms can be obtained from Mrs L. A. Henley, National Secretary, The Psoriasis Association, 7 Milton Street, Northampton NN2 7JG, U.K.

British Journal of Dermatology (1990) **122,** 435–444.

Recognition of two distinct major antigens by bullous pemphigoid sera

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SUMMARY

Sera from 17 patients with bullous pemphigoid identified a range of polypeptides of relative molecular mass (M_r) 240,000, 230,000, 190,000, 180,000, 120,000 and 100,000 from extracts of SCaBER cells, cultured human keratinocytes or human epidermis, using an immunoblotting technique. The pattern of polypeptides was characteristic for the patient serum and individual sera identified similar polypeptides from all three substrates. All 17 sera recognized major polypeptides of either M_r 230,000 (11 sera) or M_r 180,000 (seven sera) under the denaturing conditions used for immunoblotting studies. Sera from 12 patients were also examined using an immunoprecipitation technique. Polypeptide(s) of M_r 230,000 were immunoprecipitated from extracts of SCaBER cells by 11 of these sera, despite immunoblotting patterns of M_r 180,000 (or less) for three of the 11 sera. None of the minor polypeptides recognized in immunoblotting studies were immunoprecipitated by these sera. Localization of antigens was determined by binding of sera to intact or permeabilized SCaBER cells in an ELISA. Sera which recognized the M_r 230,000 polypeptide under denaturing conditions also identified an intracellular epitope in SCaBER cells, while sera which identified the denatured M_r 180,000 polypeptide bound to a cell surface epitope.

Two distinct major antigens are recognized by bullous pemphigoid sera. These both appear as molecules of M_r 230,000 under non-denaturing conditions, but only one of the molecules is dissociated to produce a M_r 180,000 polypeptide under denaturing conditions. Epitopes on these two major antigens are localized on either side of the cell membrane.

Bullous pemphigoid is an autoimmune blistering disease in which antibodies and complement are fixed to the basement membrane zone (BMZ) of epidermis. Circulating anti-BMZ antibodies can be detected by indirect immunofluorescence (IIF) in at least 70% of patients.[1] Using immunoelectron microscopy techniques, antigens have been localized to both extra-

Correspondence: Dr A.L.Cook.

cellular and intracellular sites, at the BMZ[2] or the cytoplasmic face of hemidesmosomes of the basal cells.[3,4]

A range of polypeptides is recognized by sera from patients with bullous pemphigoid. Polypeptides of relative molecular mass (M_r) 77,000–240,000,[5] or M_r 220,000–240,000,[6] were identified in extracts of human epidermis or rabbit corneal epithelium,[7] using immunoblotting techniques. In contrast, a single antigen[3,8] or doublet[6] of M_r 220,000–240,000 was identified from extracts of cultured keratinocytes, using immunoprecipitation techniques. It is not clear whether these discrepancies are due either to the source of antigens, or to recognition of denatured antigens in immunoblotting studies and native antigens by immunoprecipitation techniques.

In this study, polypeptides identified by sera from patients with bullous pemphigoid were characterized using both immunoblotting and immunoprecipitation techniques. A squamous carcinoma cell line, SCaBER, cultured normal human keratinocytes (NHK) or human epidermis were used as substrates. The SCaBER cell line has previously been used to study antibodies in patient sera which are directed against extracellular or intracellular epitopes.[9]

METHODS

Cell lines

The SCaBER cell line was derived from a squamous cell carcinoma of human bladder.[10] The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/l L-glutamine and 20 mmol/l HEPES buffer. Confluent cells were subcultured every 3–4 days using 0·25% trypsin and 0·1% ethylenediamine tetraacetic acid (EDTA) in calcium and magnesium-free Hanks' balanced salt solution, before reseeding at 1×10^6 cells per 80 cm^2 flask.

Human keratinocytes were cultured from skin obtained after surgery. The epidermis was separated from the dermis by incubation with 0·125% dispase for 16 h at 4°C and epidermal cells were dissociated by incubation with 0·05% trypsin and 0·02% EDTA for 30 min at 37°C. The cells were seeded at 10^5 per cm^2 in 3 parts DMEM and 1 part Ham's F-12 supplemented with 10% FCS, 2 mmol/l L-glutamine, 1·1 μmol/l hydrocortisone, 0·1 pmol/l cholera toxin and 20 mmol/l HEPES buffer. After the cells had plated down, 1·64 pmol/l human epidermal growth factor was added to the medium. Cells were subcultured when almost confluent and reseeded at a 1:2 split ratio.

A fibroblast cell line was established from outgrowth cultures of human dermis, using standard techniques.

Sera

Sera were taken from 17 patients with bullous pemphigoid. The diagnosis was determined by clinical, histological and immunopathological criteria. Two patients had mucous-membrane involvement and four patients had localized disease. All sera were positive, determined by IIF on normal human skin sections, with titres from 1/40 to 1/640.

Control sera were taken from 12 individuals with unrelated inflammatory dermatoses and from six patients with pemphigus vulgaris. Sera from patients with pemphigus vulgaris had titres of 1/40–1/1280. All the sera were stored at −20°C.

Preparation of cell extracts

Extracts of SCaBER cells, cultured keratinocytes, fibroblasts or human epidermis were prepared according to the method of Labib *et al.*[5] Heat-separated epidermis was homogenized in 0·22 ml extraction buffer per cm^2. Confluent layers of cultured cells were scraped into 0·05 ml extraction buffer per cm^2 and sonicated for 3 × 10 s at 4°C. The extraction buffer comprised 35 mmol/l sodium dodecyl sulphate (SDS), 0·64 mol/l 2-mercaptoethanol, 2 mmol/l phenylmethyl sulphonyl fluoride (PMSF), 10 mmol/l EDTA, 15 μmol/l pepstatin, 21 μmol/l leupeptin, 15 μmol/l antipain and 16 μmol/l chymostatin. Extracts were heated at 100°C for 3 min, cooled on ice and centrifuged at 100,000 ***g*** for 30 min at 4°C. The supernatants were stored at −70°C.

A total of five extracts were prepared from SCaBER cells (passage numbers 88–98), five extracts from NHK, three extracts from human epidermis and one extract from a fibroblast cell line.

Immunoblotting

Immunoblotting was performed by a modification of the methods of Labib *et al.*[5] and Stanley *et al.*[6] A 30–40 μl sample of cell extract was diluted with an equal volume of gel sample buffer, separated on SDS-polyacrylamide gels and transferred onto nitrocellulose sheets. The nitrocellulose was blocked with 3% bovine serum albumin (BSA) for 1 h at 37°C and incubated for 3 h at 37°C with 1/100 or 1/200 dilutions of patient or control sera in 10 mmol/l Tris buffer pH 7·6, 0·15 mol/l NaCl (TBS) with 3% BSA and 0·5% Nonidet P-40. The nitrocellulose sheets were washed using six changes of TBS and 0·5% Nonidet P-40, for 1 h. Antibody binding was detected by incubation with a 1/1000 dilution of horseradish peroxidase-conjugated rabbit anti-human IgG (Dako) for 1 h at 37°C. After washing in four changes of TBS and 0·5% Nonidet P-40 and two changes of 50 mmol/l Tris buffer at pH 7·6, antibody binding was visualized using 0·14 mmol/l 3,3′ diaminobenzidine tetrahydrochloride and 3 mmol/l hydrogen peroxide in 50 mmol/l Tris/HCl at pH 7·6.

Immunoprecipitation

Confluent SCaBER cells or NHK were extracted with 25 μl of phosphate-buffered saline pH 7·4, 0·5% Nonidet-P40, 2 mmol/l EDTA and 1 mmol/l PMSF per cm^2 of cultured cells. Cell extracts were mixed with 0·1 M sodium borate buffer pH 8·5, before radiolabelling with 40 μCi of Bolton and Hunter reagent per ml. Radiolabelled cell extracts were pre-absorbed with 20 μl of control serum (C1), prior to immunoprecipitation with 20 μl of patient or control serum per ml of cell extract, according to the method of Stanley *et al.*[8] Antigen–antibody complexes were dissociated by boiling in 100 μl of gel sample buffer containing 2-mercaptoethanol. The polypeptides were separated by SDS-PAGE, as described above. Autoradiography was performed for 4–7 days at −70°C.

ELISA

Binding of bullous pemphigoid sera to intracellular or extracellular epitopes was determined using viable or permeabilized SCaBER cells as substrate for an ELISA.[9] Specific binding is indicated by the ELISA binding index, which represents the optical density (O.D.) at 492 nm for a 1/256 dilution of test serum, after subtraction of the background binding calculated using a similar dilution of reference control serum.

RESULTS

Immunoblotting

Sera from 17 patients with bullous pemphigoid were selected on the basis of positive IIF-staining on human skin sections. A range of polypeptides was identified by all 17 sera tested, which are therefore considered to be positive by immunoblotting. Polypeptides of M_r 240,000, 230,000, 190,000, 180,000, 120,000, 100,000 or 80,000 were identified in extracts of SCaBER cells (Fig. 1a), cultured human keratinocytes (Fig. 1b) or human epidermis (Fig. 2a). These polypeptides were not identified in any cell extracts by 18 control sera, nor in extracts of cultured human fibroblasts by bullous pemphigoid sera (Fig. 2b). Non-specific binding of patient and control sera was observed with polypeptides of less than M_r 80,000.

Individual bullous pemphigoid sera recognized a similar set of polypeptides from consecutive cell extracts and also from extracts of SCaBER cells, cultured keratinocytes or human epidermis, in the majority of cases. In two cases, however, patient sera recognized a doublet of M_r 240,000–230,000 from SCaBER cells and cultured keratinocytes, compared with a single polypeptide of M_r 230,000 from human epidermis.

The number and range of polypeptides recognized by 17 bullous pemphigoid sera are illustrated in Figure 3. All sera recognized polypeptides of M_r 230,000 (10 sera) or 180,000 (six sera) and one patient serum recognized both these polypeptides.

Additional serum samples obtained from three patients were IIF-negative on skin sections, but showed identical immunoblotting patterns to the IIF-positive serum samples from these individuals. Thus, immunoblotting may be a more sensitive method for detection of specific antibodies in patient serum.

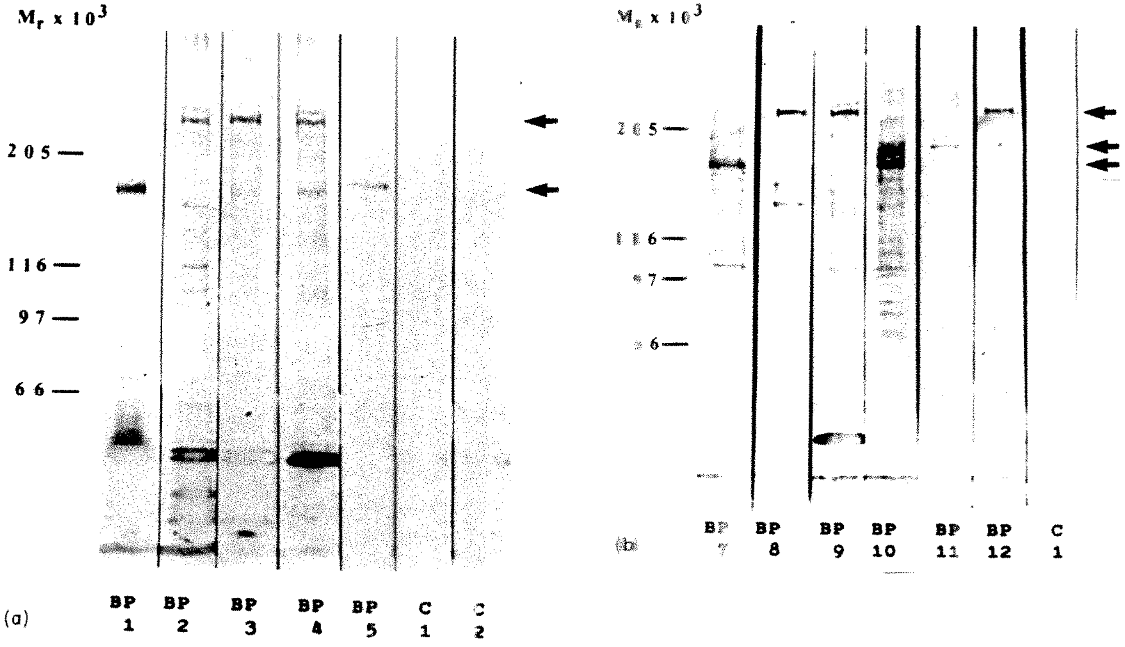

FIGURE 1. Polypeptides identified by bullous pemphigoid sera (BP) or control sera (C) in extracts of (a) SCaBER cells or (b) cultured human keratinocytes. Components of the cell extracts were separated by SDS-PAGE, transferred to nitrocellulose sheets, and stained using the immunoperoxidase method. Bullous pemphigoid sera recognized polypeptides of M_r 240,000, 230,000, 190,000 and 180,000. Migration of M_r standards are indicated.

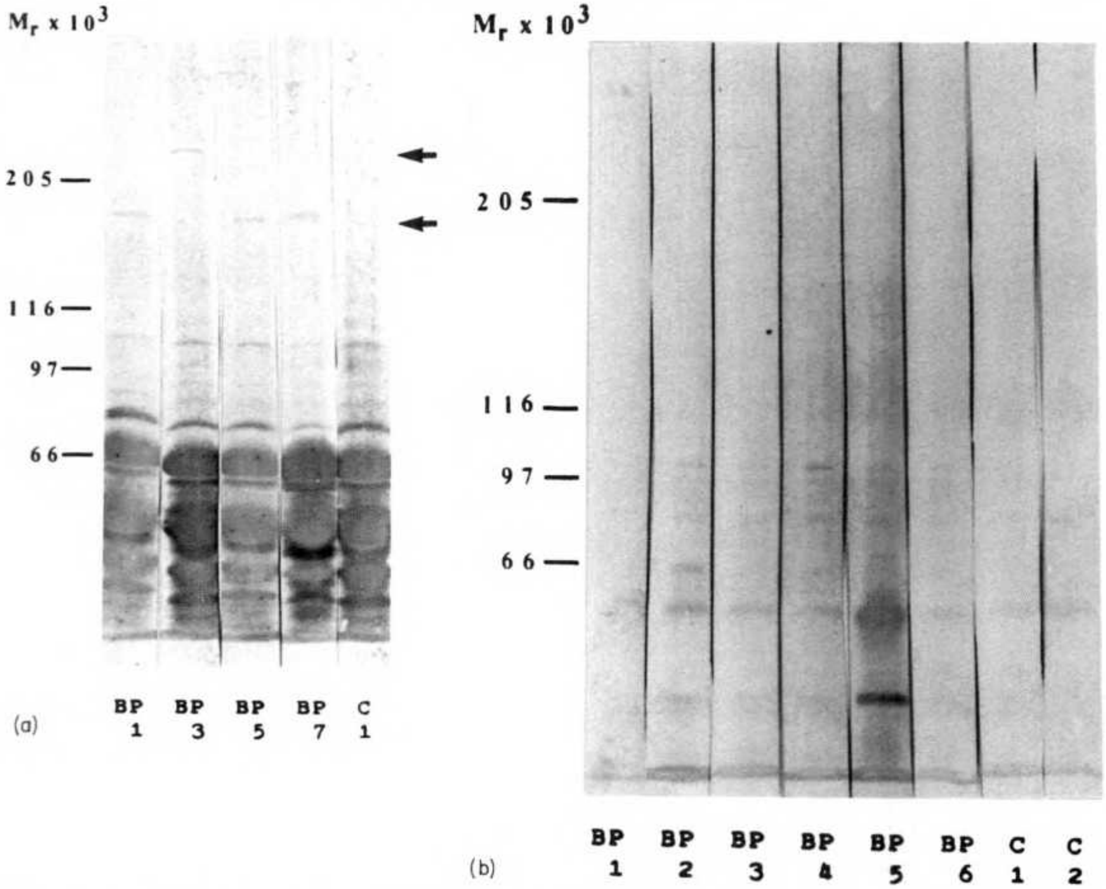

FIGURE 2. (a) Polypeptides recognized by bullous pemphigoid sera (BP) or control sera (C) in extracts of human epidermis, using an immunoblotting technique. Bullous pemphigoid sera identified polypeptides of M_r 230,000 or M_r 180,000. (b) Non-specific binding of sera, demonstrated using extracts of cultured human fibroblasts.

Immunoprecipitation

Sera from 12 patients with bullous pemphigoid were selected for immunoprecipitation studies. These sera had immunoblotting patterns of M_r 230,000 (seven sera), M_r 180,000 (four sera), or both major polypeptides (one serum), in addition to minor polypeptides. Sera from 11 patients immunoprecipitated a polypeptide of M_r 230,000 from radiolabelled extracts of SCaBER cells (Fig. 4a). A similar polypeptide was also immunoprecipitated by five of six patient sera from extracts of cultured keratinocytes, confirming the results with SCaBER cell extracts. Control sera from seven individuals and from three patients with pemphigus vulgaris did not immunoprecipitate the M_r 230,000 polypeptide (Fig. 4b).

One patient serum did not immunoprecipitate polypeptides from either SCaBER cell or NHK extracts, although this serum recognized a M_r 180,000 polypeptide in immunoblotting studies. This patient serum had a low initial IIF-titre of 1/40 and had been stored over 6 years at −40°C before use in immunoprecipitation studies.

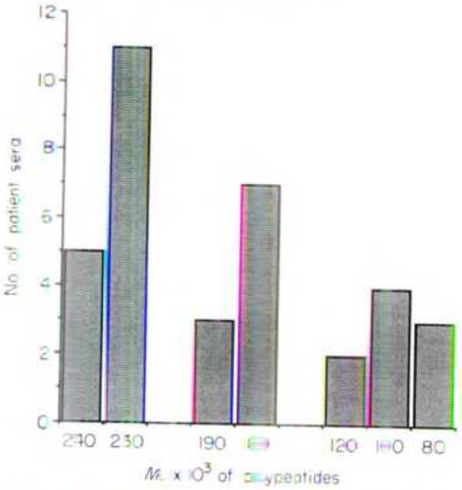

FIGURE 3. Number and range of polypeptides recognized by sera from 17 patients with bullous pemphigoid in extracts of SCaBER cells, cultured human keratinocytes or human epidermis.

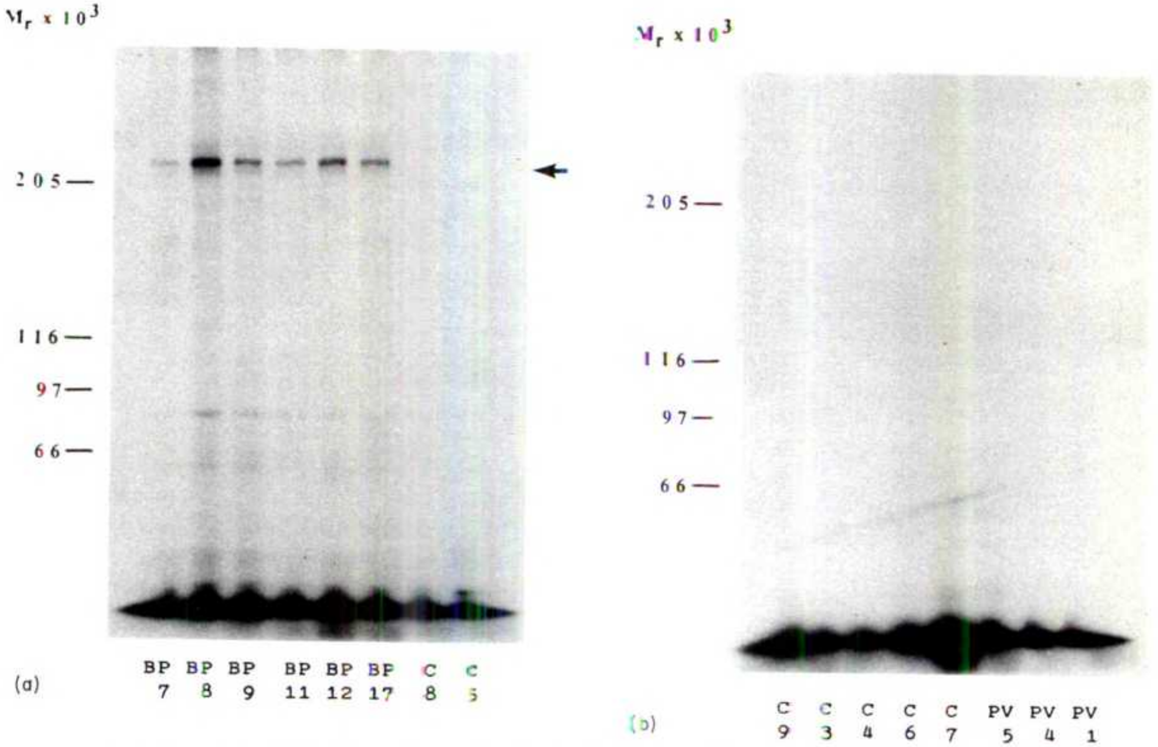

FIGURE 4. Polypeptides immunoprecipitated by (a) bullous pemphigoid sera (BP) or (b) control sera (C), including sera from patients with pemphigus vulgaris (PV), from extracts of SCaBER cells. Bullous pemphigoid sera immunoprecipitated a radiolabelled polypeptide of M_r 230,000, determined by autoradiography. Migration of M_r standards are indicated.

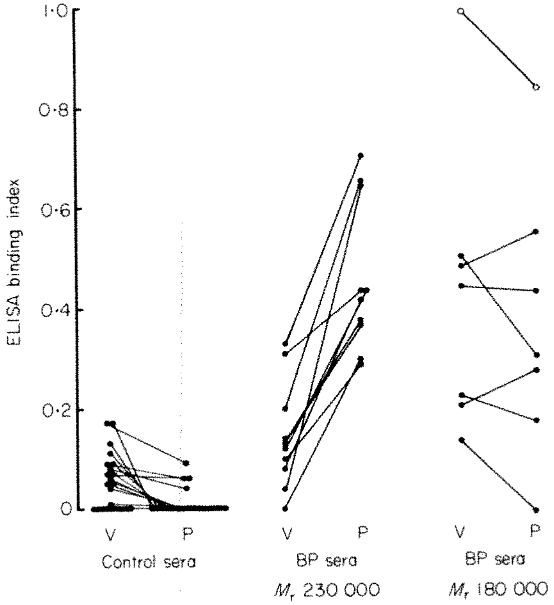

FIGURE 5. Differences in specific binding of bullous pemphigoid sera (BP) to intact, viable SCaBER cells (V) or permeabilized cells (P), determined by ELISA. Bullous pemphigoid sera which recognize the M_r 230,000 polypeptide in immunoblotting studies showed a significant increase in specific binding to permeabilized cells ($P < 0{\cdot}01$). Sera which recognize the M_r 180,000 polypeptide showed similar specific binding to both viable and permeabilized cells.

Localization of antigens

Antigens were localized by binding of bullous pemphigoid serum to intact, viable SCaBER cells or permeabilized cells, determined by ELISA. Sera were grouped according to specificity for either the M_r 230,000 or 180,000 polypeptide, in immunoblotting studies. Sera which recognized the M_r 230,000 polypeptide bound predominantly to permeabilized cells (Fig. 5). This difference in specific binding to viable or permeabilized SCaBER cells was significant at $P < 0{\cdot}01$ (Wilcoxon signed rank test) for these sera. In contrast, sera which identified the M_r 180,000 polypeptide showed comparable specific binding to both viable and permeabilized cells (Fig. 5). One patient serum recognized both major polypeptides in immunoblotting studies, but bound beyond the limits of the ELISA (Fig. 5, open circles).

DISCUSSION

The range of polypeptides from M_r 80,000 to 230,000 recognized by sera from patients with bullous pemphigoid appear to be specific for the disease, since they are not recognized by a panel of control sera, nor identified in extracts of cultured human fibroblasts. All patient sera recognized either or both of the major polypeptides in extracts of SCaBER cells, cultured keratinocytes or epidermis. The M_r 230,000 polypeptide was identified by 59% of sera, the M_r 180,000 polypeptide by 35% of sera and both polypeptides by 6% of sera. A similar range of polypeptides from M_r 77,000 to 240,000 has been identified in a previous immunoblotting study,[5] with the majority of bullous pemphigoid sera recognizing polypeptides of M_r 230,000 and 180,000, although only the M_r 230,000 polypeptide has been detected in extracts of human skin by some authors.[6,11] These discrepancies may be explained by the differences in buffers and protease inhibitors used for preparation of the cell extracts.

The pattern of polypeptides identified by individual sera suggests an association between certain polypeptides. For example, 57% of bullous pemphigoid sera which recognized the M_r 180,000 band also identified bands at M_r 100,000 and/or 80,000, which appear to be cleavage products of the major M_r 180,000 polypeptide. The M_r 180,000 polypeptide, however, is unlikely to be derived from the M_r 230,000 polypeptide, since antisera which recognize the smaller polypeptide would also recognize the larger polypeptide in the cell extracts. This is observed with only one patient serum, and the remaining 16 patient sera identify either the M_r 230,000 or the M_r 180,000 polypeptide.

Since individual patient sera recognized a characteristic set of polypeptides in extracts of either cultured cells (SCaBER cells or NHK) or human epidermis, discrepancies between antigens identified from human skin[5] or from cultured keratinocytes[3,8] cannot be attributed to differences in antigen expression *in vivo* or *in vitro*. To examine whether these differences are due to recognition of denatured or native antigens by bullous pemphigoid sera, polypeptides were immunoprecipitated from extracts of SCaBER cells or cultured keratinocytes prepared under non-denaturing conditions. All but one patient serum immunoprecipitated a major polypeptide of M_r 230,000, although three of these patient sera recognized only the major M_r 180,000 polypeptide under denaturing conditions. Thus, bullous pemphigoid sera recognize native antigen(s) of M_r 230,000, but also identify two distinct denatured antigens of M_r 230,000 and M_r 180,000. The epitopes recognized by patient sera are likely to be present on different antigens, since the majority of sera which recognized the denatured M_r 180,000 polypeptide did not recognize the M_r 230,000 polypeptide under these conditions. Further evidence for the existence of two separate major antigens is the detection of only the M_r 180,000 antigen in extracts of human amnion by bullous pemphigoid sera which identify both the M_r 230,000 and 180,000 antigens in extracts of human epidermis.[12]

Localization of these distinct epitopes was demonstrated by binding of patient sera with a particular specificity to either viable or permeabilized SCaBER cells in an ELISA. An increase in specific binding to permeable cells, compared with viable cells, indicates recognition of intracellular epitopes. Patient sera which show similar specific binding to both viable and permeabilized cells must recognize extracellular epitopes. Bullous pemphigoid sera which

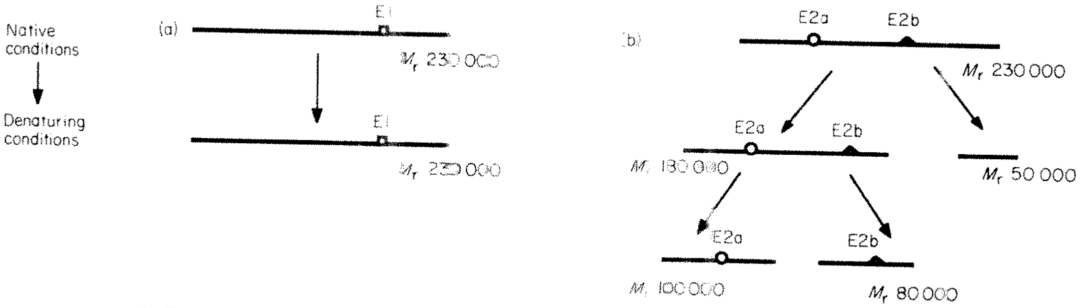

FIGURE 6. Proposed model for the structure of two distinct bullous pemphigoid antigens. Bullous pemphigoid sera recognize epitopes (E1, E2a or E2b) on either or both of the major antigens. Denaturation of the antigens under the conditions used for immunoblotting studies allows discrimination between two native antigens of M_r 230,000.

recognized the M_r 180,000 polypeptide in immunoblotting studies also identified extracellular epitope(s) and those specific for the M_r 230,000 polypeptide identified intracellular epitope(s). It appears that heterogeneous antibodies in sera from patients with bullous pemphigoid recognize at least two distinct epitopes, which are localized at either side of the cell membrane. This hypothesis would explain the detection of antigens in the BMZ[2] or within basal cells,[3,4] using different patient sera as antigen probes.

The distinct epitopes recognized by bullous pemphigoid sera appear to be present on two separate M_r 230,000 antigens, which cannot be distinguished in native conditions by immunoprecipitation studies (Fig. 6). One of the epitopes (E1) is recognized in either a native or denatured configuration (Fig. 6a). The other epitope(s) are present on a distinct antigen which exists at M_r 230,000 under native conditions, but is dissociated to produce a molecule of M_r 180,000 under conditions used for some immunoblotting studies (Fig. 6b). Although these proteins are of similar size under native conditions, the difference in stability under denaturing conditions shows that they are distinct proteins. Heterogeneous antibodies present in patient sera appear to be directed against either of these antigens, with no cross-reactivity detectable by immunoblotting. These results are confirmed by a recent report,[13] describing affinity purification of antibodies from patient serum using either denatured M_r 230,000 or 180,000 polypeptides. Only the specific antigen is detected using an immunoblotting technique, although antibodies directed against the denatured M_r 180,000 polypeptide also immunoprecipitate the M_r 230,000 polypeptide,[13] which may indicate partial cleavage of the second antigen.

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Western blot analysis of the antigen in pemphigoid gestationis

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SUMMARY

Using an immunoblotting technique, sera from 25 patients with pemphigoid gestationis were examined and tested against epidermal and dermal extracts of normal skin. The major antigen recognized by seven patients' sera was a molecule of 180 kDa, pemphigoid gestationis antigen, extractable only from the epidermis. Sera from 18 patients with bullous pemphigoid were studied as positive controls and the major antigen recognized was a larger molecule of 220 kDa. There was some degree of shared recognition of antigens with three patients with pemphigoid gestationis recognizing the 220 kDa bullous pemphigoid antigen. In addition one bullous pemphigoid serum recognized the 180 kDa pemphigoid gestationis antigen. The dominant pemphigoid gestationis antigen, however, differs from bullous pemphigoid antigen.

Pemphigoid gestationis (PG) is a rare autoimmune condition characterized by subepidermal blister formation.[1] Tissue damage appears to be mediated by an IgG antibody (PG factor) directed against a constituent of the cutaneous basement membrane zone (BMZ).[2] PG factor is of the IgG1 subclass[3] and deposition of PG factor and subsequent activation of complement is integral to bulla formation.[4] The BMZ component against which PG factor is directed is not yet fully characterized. Ultrastructural studies have demonstrated immunoreagent deposition within the lamina lucida of BMZ.[5] Indirect immunofluorescence studies using chemically (1 M NaCl) separated skin as a substrate have localized the antigen on the epidermal aspect of the cleaved tissue, on the lower pole of the basal keratinocytes,[6] a binding site similar to the binding of bullous pemphigoid (BP) sera.[7]

In the majority of patients with BP, antibodies are thought to be directed against the bullous pemphigoid antigen (BPA). This molecule has been shown to be a glycoprotein macromolecule of 220 kDa[8] by immunoprecipitation, although there is evidence of molecular heterogeneity of BP antigens by immunoblotting.[9] BPA is a normal constituent of stratified squamous

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epithelium and what makes this molecule antigenic in patients with BP is unclear. The clinical, histological and immunological similarities between PG and BP have led to speculation that BPA may also be a major antigenic determinant in PG.

Employing a Western blotting technique in the analysis of sera from 25 patients with PG, we have studied the specificity of these sera to extracts of human skin to identify the antigen recognized by PG factor.

METHODS

Sera

Serum samples were obtained from 25 patients with PG, diagnosed on the basis of typical histological and immunological findings. The serum was divided into aliquots and stored at −70°C until use. All the sera were positive for anti-BMZ IgG and IgG1 using an indirect immunofluorescence technique;[3] titres varied from 1:5 to 1:128.

Control sera

Normal human sera from 10 randomly selected blood donors with no evidence of skin disease were used as controls. In addition, 18 patients with bullous pemphigoid, diagnosed clinically and confirmed by typical histological and immunological findings formed a second, positive control group. All of the patients with BP had circulating anti-BMZ antibodies detected by indirect immunofluorescence using 1 M NaCl separated skin as substrate, which was bound to the epidermal aspect of the split tissue.

Tissue extracts

Fresh human skin was dissected from surgical specimens. Excess fat and connective tissue were removed and the specimens were cut into pieces of approximately 5 × 3 mm. These were incubated overnight in 1 M NaCl solution containing 50 μM phenyl-methyl-sulphonyl-fluoride (PMSF) and 1 mM EDTA which provided separation through the BMZ. The epidermal and dermal preparations thus obtained were extracted separately using the same techniques. They were washed briefly in distilled water and incubated overnight in urea buffer (8 M urea containing 1 mM EDTA, 50 μM PMSF and 0·3M β-mercaptoethanol). The extracts were then filtered through a coarse nylon filter and centrifuged. The supernatant was divided into aliquots and stored at −20°C.

Sodium dodecyl sulphate—polyacrylamide gel electrophoresis

Tissue extract samples were prepared for electrophoresis by the addition of sodium dodecyl sulphate (SDS) sample buffer (0·08 M Tris HCl, pH 6·8; 0·1 M dithiothrieol; 10% SDS; 50% glycerol) to epidermal and dermal supernatants in a ratio 1:1. Standard slab gel SDS polyacrylamide gel electrophoresis (SDS PAGE) was performed at acrylamide concentration 5% as described by Laemmli.[10] Commercially available prestained marker proteins of MW 90 and 200 kDa were run for calibration.

Western blotting

Proteins separated by SDS-PAGE slab gel were transferred electrophoretically to nitrocellulose membrane using the trans-blot cell (BioRad) as described by Towbin.[11] The unoccupied protein binding sites on the nitrocellulose membrane were blocked by incubation overnight with PBS containing 3% BSA and 0·05% Tween 20. The blocked membrane was then cut into strips 4 mm wide. Each strip was incubated for 1 h at 37°C with patients' or control serum,

TABLE 1. Frequency of detection of protein bands in Western blots

Serum studied	Total in group	Number of sera detecting bands M_r 180 kDa	 M_r 220 kDa
Normal human sera	10	0	0
Bullous pemphigoid	18	1	7
Pemphigoid gestationis	25	7	3

diluted 1 : 5 with 0·05% Tween 20 in PBS at pH 7·4. After washing for 20 min in PBS/Tween 20, then 20 min PBS, each strip was incubated for 1 h with peroxidase-labelled affinity purified goat anti-human IgG (Kirkegard and Perry, MD, U.S.A.). Finally the strips were washed again as before and then visualized by incubation for 15 min at room temperature with a freshly prepared solution of 3,3′ diaminobenzidine HCl (0·05 mg/ml) and 0·01% H_2O_2 in 50 mM Tris HCl, pH 7·6. Each strip was washed in PBS and air-dried. The SDS-PAGE and Western blotting experiments were separately performed in two different laboratories. Only when both sets of results were identical on repeated testing were they interpreted as conclusive.

RESULTS

The frequency of detection of protein bands in PG, BP and control sera is summarized in Table 1. The major molecular antigenic determinant detected in the PG sera by immunoblotting was a macromolecule of 180 kDa, with seven sera of 25 tested (28%) recognizing this protein (Fig. 1). This molecule was extracted from the epidermal component of skin but could not be detected in the dermal extract. In our group of positive control patients with BP, the most consistent binding was to a protein band of 220 kDa. One bullous pemphigoid serum reacted with a molecule of 180 kDa (Fig. 1). In three PG sera (12%) the 220 kDa protein band was also recognized but always in addition to the 180 kDa band. No PG sera bound exclusively to the 220 kDa protein. The remaining 18 patients with PG did not consistently bind to either the 180 or 220 kDa protein, nor to a protein of any other molecular weight.

No normal control sera identified either the 180 kDa or the 220 kDa protein. A variety of bands of 95, 115, 125 and 135 kDa (Fig. 1) were detected in an irregular fashion in all three groups of sera. These were often of irregular density of staining and were generally of a diffuse nature. Multiple bands were also identified in the range 10–60 kDa, again of diffuse nature and with a character unlike the bands detected at 180 and 220 kDa.

DISCUSSION

The cutaneous BMZ is a complex structure composed of a variety of components.[12] Some of these components, such as type IV collagen, laminin, fibronectin, entactin and epidermolysis bullosa acquisita antigen have been fully categorized,[13-16] but others await accurate identification. In the acquired subepidermal bullous diseases autoantibodies are produced which react with an antigen associated with the BMZ. In BP the antigen has been identified as bullous pemphigoid antigen (BPA)[8] which is a macromolecule glycoprotein synthesized by the basal

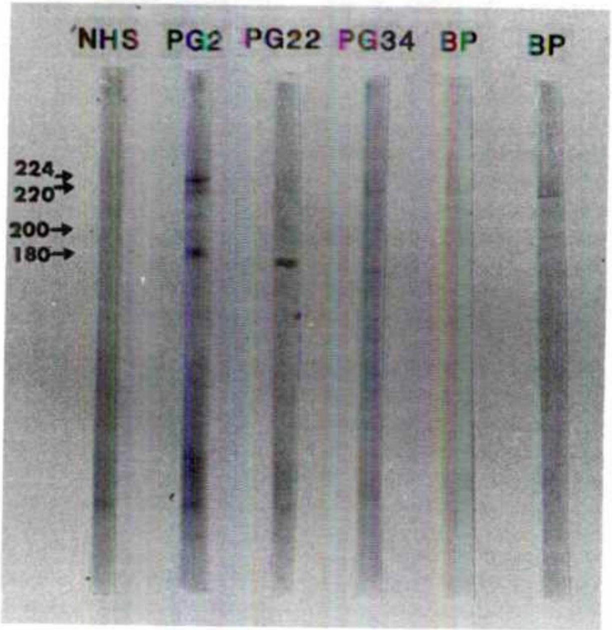

FIGURE 1. Immunoblot of epidermal extract. Pemphigoid gestationis sera, lanes 2, 3, 4 identify an epidermal antigen of 180 kDa. Pemphigoid gestationis sera in lanes 2 and 4 in addition bind to a molecule of 220 kDa. Bullous pemphigoid sera (lanes 5 and 6) bind to a molecule of 220 kDa. Normal human serum (lane 1) binds to neither of these molecules. Insignificant binding to small M_r contaminants may be seen in all the lanes (Immunoperoxidase).

keratinocyte. Most workers estimate BPA to be of the order of 220 kDa,[8] but some recent studies recognize an element of antigenic heterogeneity.[9,17]

In this study it has been shown by immunoblotting that in PG the major antigenic determinant recognized by a significant proportion of patients' sera is a macromolecule of 180 kDa. This molecule, PG antigen, can be extracted from the normal epidermis and is a normal constituent of skin. Some PG sera also bind to the major antigen in BP, which in our study is confirmed as a 220 kDa protein. Similarly, some BP sera bind to the major PG antigen in addition to BPA. There is, therefore, some degree of shared molecular specificity between the two conditions.

The exact molecular nature of PG antigen needs to be determined, particularly the nature of the antigenicity of this molecule, which is clearly non-antigenic in the normal individual. We have previously found evidence of antigenic cross reactivity between the skin and placenta in PG.[6] In the placenta, the antigen can be demonstrated from the second trimester of pregnancy until term, and it may be that exposure to this antigen during pregnancy is required for antibody production. Further evidence for placental initiation of PG comes from studies[18,19] which have demonstrated abnormal expression of MHC II molecules in the placentae of patients with PG. A similar method of triggering autoimmune responses has been demonstrated in a variety of tissue-specific autoimmune disease.[20] Local allogeneic reactions between maternal lymphocytes and paternal MHC class II molecules in the placenta may be important in the initiation of the autoimmune response in PG.[21]

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Alterations induced in normal human skin by *in vivo* interferon-gamma

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SUMMARY

In a study of the direct effects of interferon-gamma (IFN-γ) on normal human skin, healthy adult male volunteers received either 3 μg ($n=4$) or 30 μg ($n=9$) of recombinant IFN-γ administered intradermally over 3 days. Biopsies were taken on day 6 and histopathological examination of fixed paraffin-embedded sections from sites which had received 30 μg IFN-γ revealed a moderate perivascular lymphohistiocytic dermal infiltrate with mast cells. Immunophenotyping of 5 μm cryostat sections demonstrated that 3 μg IFN-γ induced keratinocyte HLA-DR expression in the absence of any significant infiltrate. More intense keratinocyte HLA-DR expression was produced by 30 μg IFN-γ in all specimens, with HLA-DP concurrently expressed in three biopsies. The ratio of CD4:CD8 cells within the infiltrate was approximately 3:1. CD1$^+$ cells within the epidermis were markedly depleted by 30 μg IFN-γ, while CD1-labelled cells were observed in the dermal perivascular infiltrate. Intradermal IFN-γ induces similar immunopathological changes to those observed in many of the inflammatory dermatoses.

Interferon-gamma (IFN-γ), a glycoprotein whose gene in the human is located on the short arm of chromosome 12, is a soluble cytokine derived from activated T lymphocytes.[1] Although it possesses both antiviral and antiproliferative activity, its main physiological function appears to be as a potent modulator of most T lymphocyte-mediated immune responses.[2] These immunomodulatory properties include: mediation of both macrophage and B lymphocyte activation, enhancement of T lymphocyte activity including natural killer cell activity, increased production of class II major histocompatibility (MHC) antigens by antigen presenting cells (APC), and induction of aberrant class II MHC antigen expression by a variety of epithelial cell types.[3]

Many inflammatory dermatoses such as lichen planus, discoid lupus erythematosus and allergic contact dermatitis, are characterized by a dermal infiltrate in which activated T

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lymphocytes are prominent, and by keratinocyte expression of HLA-DR,[4,5] one product of the class II MHC locus. Since IFN-γ is derived from activated T lymphocytes and can induce keratinocyte HLA-DR expression,[6,7] it may play an important role in the pathophysiology of these inflammatory cutaneous diseases.

To date, studies of the *in vivo* effects of IFN-γ applied locally to human skin have been confined to its effects on diseased skin. In particular, intradermally administered IFN-γ induces a number of immunopathological changes in lesional tissue of lepromatous leprosy[8,9] and in plaques of psoriasis.[10] However, since the dermal infiltrate of both conditions possesses many immunologically active cells including T lymphocytes and macrophages, the observed effects of locally administered IFN-γ may result from stimulation of secondary immunological pathways. This study was carried out to identify the histopathological and immunopathological changes induced by *in vivo* IFN-γ on normal human skin.

METHODS

Subjects

Having obtained appropriate ethical approval, 13 normal adult male volunteers were recruited (age range 18–44 years). Volunteers with a personal or family history of atopy or psoriasis were excluded, as were penicillin-sensitive subjects. Each volunteer received a general medical examination, including the measurement of pulse rate, blood pressure and temperature. Urine samples were analysed for the presence of blood, protein and sugar. Whole blood was screened for haematological and biochemical abnormalities. Any abnormal finding resulted in exclusion of the volunteer from the study.

Interferon-gamma

The study design was based on the methodology of Nathan *et al.*[8] Lyophylized recombinant human interferon-gamma (rIFN-γ) (specific activity = 1×10^7 U/mg) was donated by Biogen Incorporated (Boston, MA, U.S.A.). Each volunteer received an intradermal injection of rIFN-γ diluted in 0·1 ml sterile water in the same area on the left buttock daily for 3 consecutive days. Nine received a daily dose of 10 μg rIFN-γ (total dose = 30 μg) and four received 1 μg rIFN-γ daily (total dose = 3 μg). As a control, an area on the right buttock received the diluent only. Volunteers were questioned daily about the development of symptoms and the injection site was assessed for evidence of pain, erythema and induration.

Biopsy

Punch biopsies (6 mm) were obtained on day 6 from both active and control sites after local anaesthetic (1% lignocaine) was injected around (but not into) the biopsy site. Each skin specimen was then bisected and half was fixed in formalin prior to embedding in paraffin, and the other half embedded in OCT, snap frozen and stored in liquid nitrogen.

Histochemistry

Sections (5 μm) were obtained from the paraffin-embedded sections and plated on to glass slides. The sections were subsequently dewaxed and stained with either haematoxylin and eosin or chloroacetate esterase.

Immunohistochemistry

Cryostat sections (5 μm) were obtained from the unfixed frozen biopsies stored in liquid nitrogen. Each slide was air-dried for 10 min, fixed in acetone for 10 min and subsequently stained by the three step peroxidase anti-peroxidase (PAP) immunohistochemical assay, with diaminobenzidine as substrate.[11] Primary murine monoclonal antibodies (mAb) to a range of cell surface determinants were employed as listed in Table 1. The second layer ('bridging') antibody consisted of rabbit anti-mouse IgG, and the third layer of a peroxidase mouse anti-peroxidase complex. Using this immunohistochemical technique, positive staining was identified as a brown reaction product. All biopsies, 13 active and 13 control, were examined with each antibody listed in Table 1. When quantifying T-cell subsets, positively labelled cells in the upper dermal infiltrate for the relevant antibodies were counted using an eyepiece graticule in 10 consecutive high power fields (×25 objective) of a Leitz light microscope. CD4:CD8 ratios were calculated for each volunteer specimen. Langerhans cells were counted by noting the number of epidermal CD1$^+$ cell bodies containing nuclei in five adjacent high power fields (×40 objective) and the mean calculated. All biopsies which had received 30 μg IFN-γ ($n = 9$) and their contralateral control ($n = 9$) were assessed in this way. The means of the Langerhans cell counts in the IFN-γ treated and control groups were compared using a paired sample '*t*' test.

RESULTS

Clinical

Three volunteers who received 10 μg rIFN-γ daily for 3 days (total dose = 30 μg) developed mild erythema and induration at the active site by day 3. This persisted for 48 h and had disappeared by day 6 leaving clinically normal skin. No systemic symptoms, in particular neither malaise nor myalgia, were reported by any of the volunteers.

Histochemistry

Histological examination of sections stained with haematoxylin and eosin from sites which had received a total rIFN-γ dose of 3 μg revealed essentially normal skin with a sparse dermal perivascular lymphohistiocytic infiltrate. Almost identical findings were observed in the control biopsy specimens. Examination of biopsies from sites which had received 30 μg rIFN-γ showed that while there was no change in epidermal thickness, the nucleoli were prominent in most keratinocyte nuclei. There was no hydropic degeneration nor premature keratinization.

TABLE 1. Primary murine mAb used in study

MAb	Cluster determinant	Specificity	Source
L243	—	HLA-DR	Becton-Dickinson*
Leu 10	—	HLA-DQ	Becton-Dickinson
B7/21	—	HLA-DP	Becton-Dickinson
OKT6	CD1	Langerhans cells	Ortho†
Leu 1	CD3	Pan T cell	Becton-Dickinson
Leu 2a	CD8	Suppressor/cytotoxic T cell	Becton-Dickinson
Leu 3a	CD4	Helper T cell	Becton-Dickinson

*Becton-Dickinson, Mountain View, CA, U.S.A.
†Ortho Pharmaceuticals, Raritan, NJ, U.S.A.

Underlying these epidermal changes there was a moderate dermal infiltrate situated predominantly around the blood vessels and consisting mostly of lymphocytes with smaller numbers of histiocytes (Fig. 1). Lymphocytes were also seen throughout the dermis, but particularly along the dermo–epidermal basement membrane zone. Sections stained with chloroacetate esterase showed that mast cells were scattered throughout the infiltrate.

Immunohistochemistry

In control biopsies, epidermal staining for each class II MHC determinant was limited to cells of the acrosyringium[12] and Langerhans cells, the latter also expressing OKT6 antigen (CD1). CD1$^+$ cells within the dermis were infrequently observed. Scattered cells throughout the dermis, including dermal endothelial cells, expressed all three class II MHC antigens. T lymphocytes, both helper (CD4$^+$) and suppressor/cytotoxic (CD8$^+$), were also observed within the dermis.

In sections from sites receiving a total rIFN-γ dose of 3 μg, keratinocytes expressed HLA-DR ($n=4$). Staining was patchy and keratinocyte expression of HLA-DQ and HLA-DP was absent from all specimens. Dermal patterns of HLA-DR staining between the active (3 μg rIFN-γ) and control biopsy sites were not significantly different.

Intense staining for HLA-DR by keratinocytes throughout the thickness of the epidermis ($n=9$) was produced by 30 μg rIFN-γ (Fig. 2). Patchy expression of HLA-DP by keratinocytes was also observed in four biopsy specimens (44%), while keratinocyte expression of HLA-DQ was absent. Within the dermal infiltrate the majority of cells expressed all three class II MHC determinants. The density of CD1$^+$ epidermal dendritic cells was significantly diminished

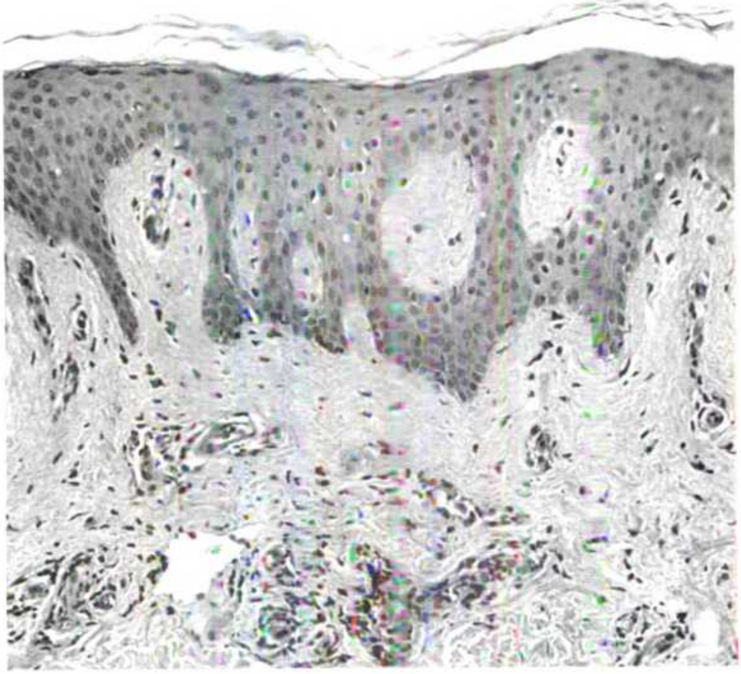

FIGURE 1. Fixed, paraffin-embedded section of skin from a volunteer who had received 30 μg rIFN-γ. There is a lymphocytic infiltrate predominantly around dermal blood vessels (H & E) (× 80)

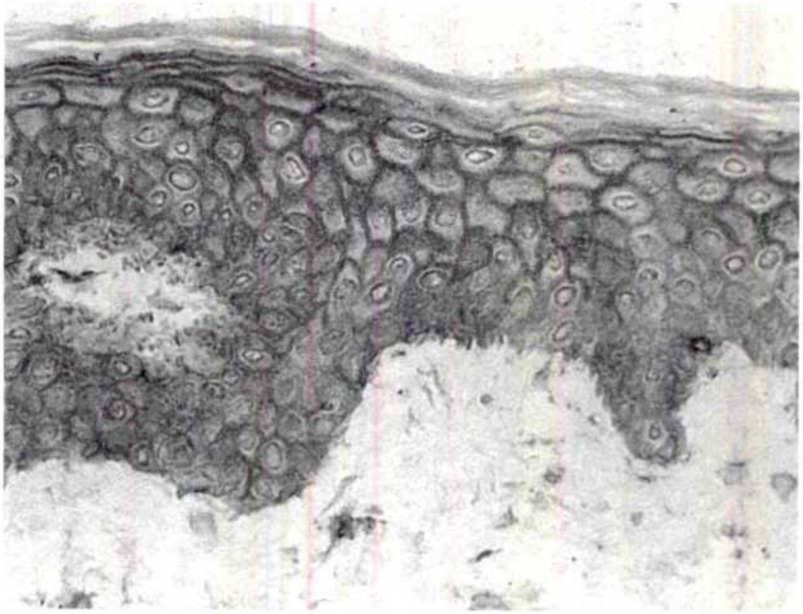

FIGURE 2. Frozen section of skin from a volunteer who had received 30 μg rIFN-γ, stained for HLA-DR. Positive keratinocyte surface labelling is seen throughout the thickness of the epidermis, giving a typical 'chicken-wire' appearance (PAP) (× 200).

($P < 0{\cdot}001$) by 30 μg rIFN-γ ($\bar{x} = 5{\cdot}25 \pm 0{\cdot}68$) (Figs 3a, b) compared with normal skin ($\bar{x} = 17{\cdot}6 \pm 1{\cdot}4$), while increased CD1 positivity was observed within the perivascular dermal infiltrate. Staining with Leu 1 revealed that 80% of the infiltrate consisted of T lymphocytes (CD3), while the CD4:CD8 ratio was 2·6:1.

DISCUSSION

This study has demonstrated that IFN-γ can induce many immunopathological changes within normal human skin. HLA-DR antigen expression was induced in keratinocytes, *in vivo*, by the action of IFN-γ, which also induced both a significant dermal infiltrate rich in T lymphocytes, and marked alterations in the distribution of $CD1^+$ cells within human skin.

Many *in vitro* studies have demonstrated that IFN-γ can induce keratinocytes to express HLA-DR.[6,7] To date, *in vivo* studies have only examined either diseased skin or the cutaneous effects of IFN-γ given systemically to patients with advanced tumours.[13] However, the source of IFN-γ in inflammatory skin disease, where keratinocytes express HLA-DR, is presumed to be activated T cells within the dermal infiltrate.[14] This implies that IFN-γ administered intradermally in healthy skin would more accurately reflect the pathophysiological events in inflammatory skin diseases. The observation that the lower doses of rIFN-γ (total dose = 3 μg) produces keratinocyte HLA-DR expression in the absence of a significant infiltrate suggests that IFN-γ, *in vivo*, acts directly on keratinocytes, presumably via a surface receptor,[15] and not via intermediate pathways. In addition, our study has shown that IFN-γ induces differential expression of class II MHC antigens by keratinoctyes, as observed *in vitro*[16] and similar to the patterns observed in inflammatory cutaneous disease.[17,18] Although the precise function of

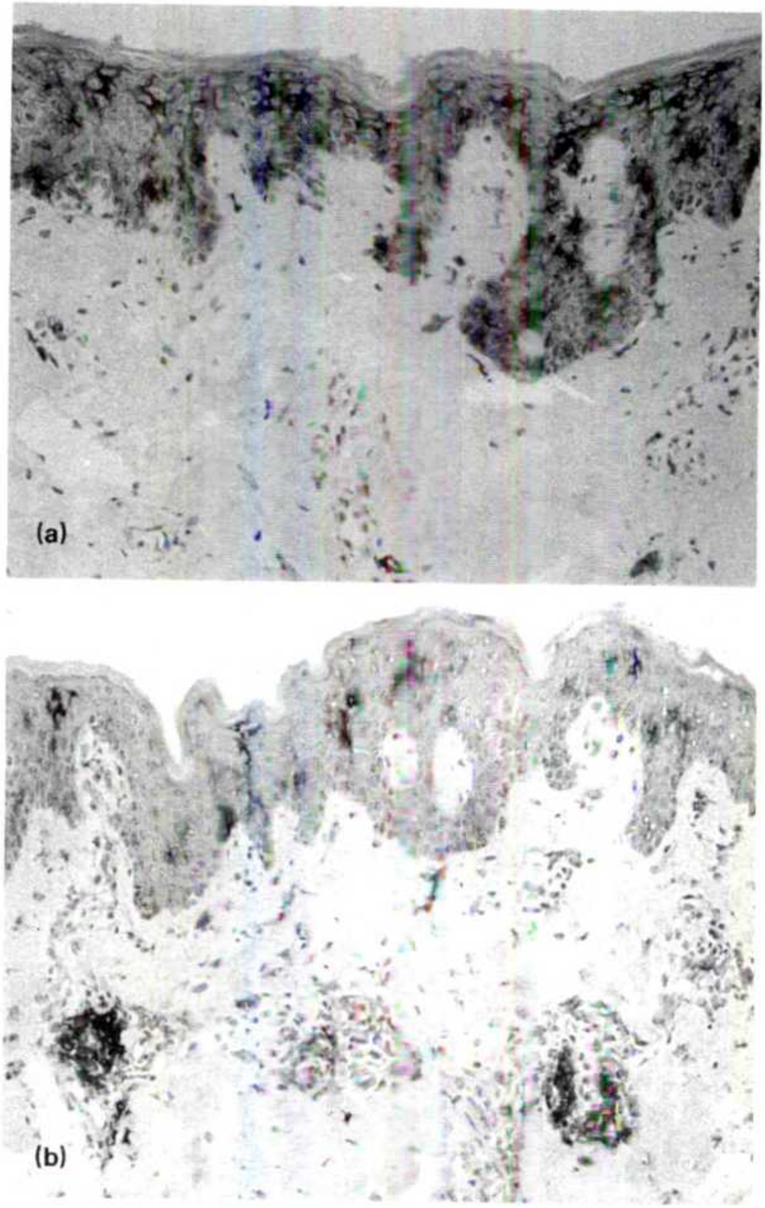

FIGURE 3. Frozen sections stained with OKT6 antibody. (a) Normal control skin, demonstrating positivity throughout the epidermis and sparse dermal staining. (b) Skin from a volunteer who received 30 μg rIFN-γ showing reduced numbers of epidermal OKT6$^+$ cells and considerable OKT6 staining within the perivascular infiltrate (PAP) (× 80).

HLA-DR$^+$ keratinocytes remains unknown, functional studies suggest that they participate in a number of immunological events. Thus, keratinocytes may both act as targets for HLA-DR-restricted cytotoxic T cells[19] and modulate cutaneous immunological reactions by inducing T-cell unresponsiveness.[20] However, evidence that HLA-DR$^+$ keratinocytes can present antigen to helper T lymphocytes is conflicting. Recently, Nickoloff has proposed varying scenarios for the immune function of IFN-γ-stimulated keratinocytes in cutaneous inflammation.[21]

Intradermally administered IFN-γ produced a mixed dermal infiltrate consisting mostly of lymphocytes but also containing histiocytes and mast cells. Such an infiltrate, mainly HLA-DR$^+$ and with a CD4:CD8 of 3:1, is also observed in many inflammatory dermatoses.[22] This suggests that, in these disorders, IFN-γ may play a role in the recruitment of circulating leucocytes into the dermis, which is further supported by the predominantly perivascular localization of the infiltrate. This is likely to involve adhesion antigens on the surface of leucocytes and their ligands on dermal endothelial cells, expression of which has been demonstrated, *in vitro*, to be modulated by several cytokines including IFN-γ.[23]

IFN-γ had a marked effect on the distribution of CD1$^+$ cells. The epidermis appeared to be depleted of Langerhans cells, while CD1$^+$ cells appeared within the perivascular infiltrate. Such a finding is also observed in allergic contact dermatitis[24] where it is thought that antigen-bearing Langerhans cells migrate from the epidermis to the dermis and, subsequently, to regional lymph nodes. The results of this study suggest that this process may be antigen independent, and modulated by cytokines within involved skin. It is also possible that Langerhans cells remain within the epidermis but assume a cytokine-induced altered-surface antigenic profile.

While other inflammatory mediators are thought to be important in the pathogenesis of inflammatory cutaneous disease, this study has demonstrated that many of the cutaneous immunophenotypic changes observed in the inflammatory dermatoses can be mimicked by intradermal IFN-γ. This suggests that IFN-γ may play a central role in the pathophysiology of many T-lymphocyte associated cutaneous diseases. Further studies are needed on the dynamic aspects of changes induced by IFN-γ and to determine the synergistic effect of IFN-γ with other cytokines.

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Epidermal adenylate cyclase system is regulated by diacylglycerol-protein kinase C signal, but not by calcium signal

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SUMMARY

The breakdown of inositol phospholipids is an important transmembrane signalling system that is composed of two kinds of signals: the diacylglycerol-protein kinase C signal, and the inositol trisphosphate-Ca^{2+} signal. Using membrane-permeable diacylglycerol, 1-oleoyl-2-acetylglycerol (OAG), and calcium ionophore, A-23187, the effects of these chemicals on the epidermal adenylate cyclase system were investigated. OAG increased forskolin- and cholera toxin-induced cyclic AMP accumulations, but receptor adenylate cyclase responses were markedly decreased by treatment with OAG. The effects of OAG were inhibited by the protein kinase C inhibitor, H-7. Calcium ionophore, A-23187, had no effect on the epidermal adenylate cyclase responses. Combinations of OAG and A-23187 (as well as the calcium chelator, EGTA), showed that the action of OAG was mostly unaffected by the modulation of intracellular and extracellular Ca^{2+} concentrations. The results suggest that among the signals triggered by the breakdown of inositol phospholipids, only diacylglycerol-protein kinase C signal is involved in the regulation of the epidermal adenylate cyclase system.

Most cells possess two major classes of receptors for the passage of information across the plasma membrane.[1] One class increases cyclic AMP through the adenylate cyclase system, which consists of at least three distinct components: receptor (R), guanine nucleotide-binding protein (G), and the catalytic component (C).[2] The other accelerates the breakdown of inositol phospholipids (PI), leading to the generation of inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).[1] IP3 mobilizes Ca^{2+} from intracellular stores and activates the Ca^{2+}-dependent processes through calcium-binding proteins such as calmodulin.[3] IP4, which is derived from IP3, is assumed to stimulate Ca^{2+} entry from the extracellular environments.[4] The other mediator, DAG, activates protein kinase C (PKC).[1,5,6] Phorbol diesters, which are potent tumour promoters, also bind to and activate PKC.[1] The most potent is phorbol,

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12-myristate, 13-acetate (PMA), which is assumed to activate PKC by substituting for the endogenous activator, DAG.[1]

Stimulatory receptors of adenylate cyclase have been well established in the epidermis.[7] These are β-adrenergic-, prostaglandin E-, adenosine-, and histamine (H2)-receptors. The physiological signal for PI breakdown in the epidermis remains unknown but recent evidence suggests that signal transducing systems are markedly altered in pathological conditions of the epidermis.[8-11] The interaction between these signal transducing systems was studied in the epidermis. The adenylate cyclase system was shown to be modulated by PMA, the effect of which was inhibited by the PKC inhibitor, H-7.[12] Although this suggests that PMA modulates the epidermal adenylate cyclase system through PKC, it has been reported that there may be a PMA effect which is not mediated by PKC.[13,14] Also, the effect of Ca^{2+}, (another major signal triggered by the PI breakdown) on the epidermal adenylate cyclase system remains unknown.

As PMA directly activates PKC, the calcium ionophore, A-23187, can substitute for the Ca^{2+} signal by opening the Ca^{2+} gate across the plasma membrane[15] or, more likely, across the endomembrane of intracellular Ca^{2+} store.[16] In this study, using a more physiological membrane-permeable DAG analog, 1-oleoyl-2-acetylglycerol (OAG)[1,17] and A-23187, the effect of these chemicals on the epidermal adenylate cyclase responses was investigated. As well as the adenylate cyclase-receptor agonists, forskolin and cholera toxin, which work on C and G respectively,[9] were used to monitor the latter part of the epidermal adenylate cyclase activity.

METHODS

Pure epidermal sheets

Domestic pigs weighing 5–10 kg were anaesthetized with Nembutal (Dainippon Pharmaceutical, Osaka, Japan) i.p. (30 mg/kg). Fifteen minutes after anaesthesia, skin slices were taken from the backs of pigs using a Castroviejo keratome (Storz Instrument Co., St Louis, MO, U.S.A.) set at 0·3 mm. The skin slices were cut into 5 × 5 mm squares and floated with their keratin layers up, in RPMI 1640 medium with 1000 U/ml of dispase (Godo Shusei, Tokyo, Japan). After incubation at 37°C in an atmosphere of 5% CO_2 in air for 30 min, pure epidermal sheets were peeled from the dermis with sharp forceps and floated in a fresh RPMI 1640 medium. These pure epidermal sheets retained, intact, the adenylate cyclase system, which has been previously described.[18]

Forskolin- and cholera toxin-induced cyclic AMP accumulations

The experimental procedure was essentially as previously described.[9] Pure epidermal sheets were incubated with 200 μM forskolin or 100 μg/ml cholera toxin in RPMI 1640 medium for the indicated time with added antibiotics (100 U/ml penicillin, 0·1 mg/ml streptomycin, 0·25 μg/ml fungizone) as well as the various chemicals (OAG, PMA, A23187, etc.) to be tested. The cyclic AMP phosphodiesterase inhibitor, 1 mM 3-isobutyl-1-methylxanthine (IBMX), was added to the incubation medium throughout the incubation. Incubations were performed at 37°C in an atmosphere of 5% CO_2 in air. The epidermal sheets were incubated for 1 and 3 h for the forskolin and cholera toxin experiments respectively, preliminary experiments having shown that these conditions resulted in the most marked cyclic AMP accumulations in the epidermis. As well as C activation, forskolin, at lower concentrations, also interacts with G and/or R of adenylate cyclase.[19] At higher concentrations such as those used in the present study (200 μM), forskolin-induced cyclic AMP accumulation is assumed to monitor the C function of adenylate cyclase.[20] Forskolin was dissolved in ethanol and the final concentration of ethanol in the incubation medium was 0·5% v/v. Cholera toxin is freely soluble in water and was directly added to the incubation medium. OAG and PMA were dissolved in dimethylsulphoxide (DMSO) and the final concentration of DMSO in the incubation medium was less than 0·2% v/v. A-23187 was dissolved in ethanol and the final concentration of ethanol was 0·2% v/v. Appropriate concentrations of ethanol and DMSO were added to the control incubation medium. Following incubation, two of the epidermal sheets were frozen between two plates of dry ice.

Receptor–adenylate cyclase responses

Pure epidermal sheets were incubated with various chemicals (OAG, PMA and A-23187) for 7 h in RPMI 1640 medium at 37°C in 5% CO_2 in air and with antibiotics. Following the 7 h incubation, two of the epidermal sheets were incubated with 50 μM epinephrine or 1 mM histamine, respectively, in the presence of 1 mM IBMX. These concentrations were sufficient for maximal accumulation of cyclic AMP.[7] Following 5 min incubation at 37°C with these receptor–adenylate cyclase agonists, the epidermal sheets were frozen in dry ice.

Cyclic AMP assay

The cyclic AMP content in the epidermal sheets was measured by radioimmunoassay using a Yamasa cyclic AMP assay kit (Yamasa Shoyu Co., Tokyo, Japan) as previously described.[9] Protein concentration was measured according to the method of Lowry *et al.*[21] with bovine serum albumin as a standard. The statistical significance of the data obtained was evaluated by Student's *t*-test.

Chemicals

1-Oleoyl-2-acetylglycerol, A-23187, and forskolin 7-deacetyl-7-*o*-hemisuccinic acid were obtained from Calbiochem (La Jolla, CA, U.S.A). PMA and cholera toxin were obtained from Sigma (St Louis, MO, U.S.A.). H-7 (1-(5-isoquinolinesulphonyl)-2-methyl piperazine dihydrochloride) was purchased from Seikagaku Kogyo Co. (Tokyo, Japan). All other chemicals were purchased from Nakarai Chemicals Ltd (Kyoto, Japan).

RESULTS

The addition of various concentrations of OAG to the incubation medium resulted in increased forskolin- and cholera toxin-induced cyclic AMP accumulations after 1–3 h of incubation (Fig. 1a). However, receptor–adenylate cyclase responses were significantly decreased by the OAG treatment (Fig 1b) after 6–7 h of incubation. Epidermis which had been pretreated with OAG for 7 h revealed a similar increase in forskolin- and cholera toxin-induced cyclic AMP accumulations. Although the effect of OAG on forskolin and cholera toxin effects were the opposite of those on the receptor adenylate cyclase responses, the effects were observed at a similar concentration range of OAG: the maximal effect was observed at 150 μg/ml.

The addition of PKC inhibitor, H-7, to the incubation medium resulted in the inhibition of the OAG effects. The OAG-induced augmentation of the forskolin effect was inhibited by H-7 (Fig. 2a). The OAG-induced augmentation of the cholera toxin effect was also inhibited by H-7 (data not shown). Also the addition of H-7 to the incubation medium resulted in increased receptor–adenylate cyclase responses of the epidermis, which was not affected by the presence of OAG (Fig. 2b).

It has been reported that PMA has similar effects on the epidermal adenylate cyclase system.[12] Comparison of the effects of OAG and PMA showed that the OAG effects were

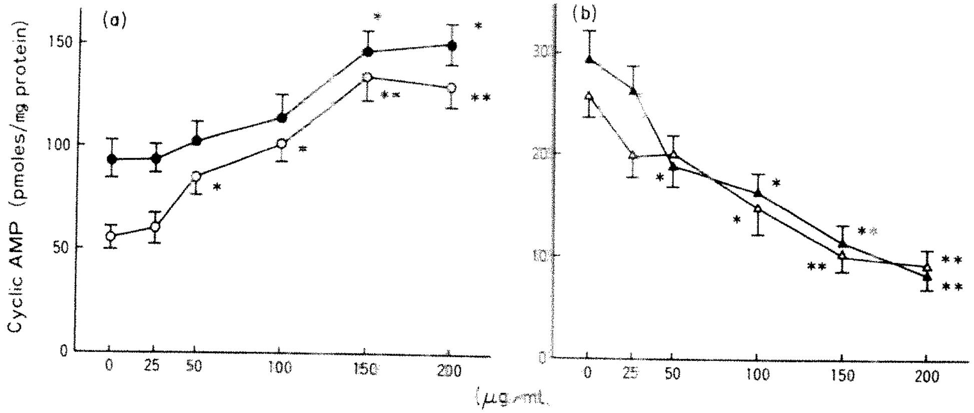

FIGURE 1. Effects of OAG on epidermal adenylate cyclase responses. (a) Pig epidermal sheets were incubated with various concentrations of OAG in the presence of 200 μM forskolin (○——○) or 100 μg/ml cholera toxin (●——●) for 1 h and 3 h, respectively. IBMX (1 mM) was added to the incubation medium throughout the incubation. (b) Pig epidermal sheets were incubated with various concentrations of OAG for 7 h. Following the incubation, epinephrine (△——△), and histamine (▲——▲)-induced cyclic AMP accumulations were measured in the presence of 1 mM IBMX.

Data are expressed as cyclic AMP pmoles/mg protein. The basal levels of cyclic AMP were all around 1 pmole/mg protein. All $n = 4$ from a typical experimental series. Bars, SE. * $= P < 0{\cdot}05$ compared with control; ** $= P < 0{\cdot}01$ compared with control.

generally stronger than the PMA effects (Table 1). The addition of both OAG and PMA at their maximally effective concentrations resulted in neither an additive nor a synergistic effect on the epidermal adenylate cyclase responses (Table 1).

In contrast to OAG, Ca^{2+} ionophore, A-23187, which increases intracellular Ca^{2+} concentrations, had no effect on the epidermal adenylate cyclase responses (Fig. 3). Also A-23187 did not have any effect on the OAG-induced modulation of epidermal adenylate

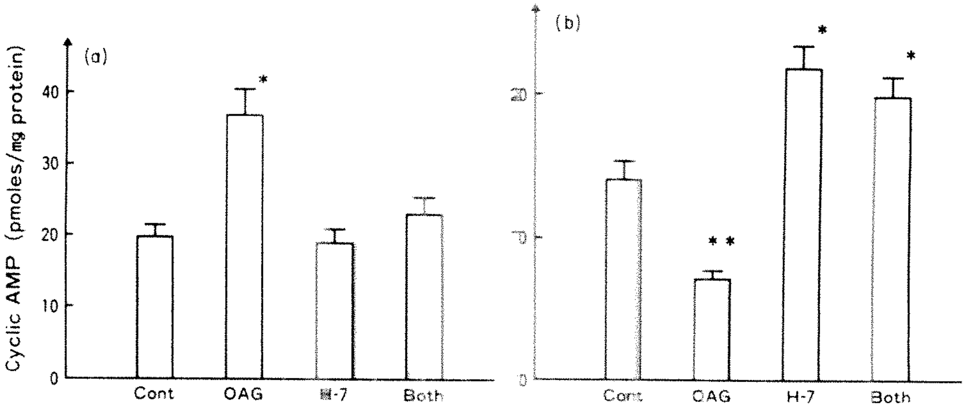

FIGURE 2. Inhibition of the OAG effects by H-7. Pig epidermal sheets were incubated with 100 μg/ml OAG, 100 μM H-7, or both compounds. Other conditions were the same as in Figure 1. (a) Forskolin-induced cyclic AMP accumulations; (b) epinephrine-induced cyclic AMP accumulations. All $n = 4$ from a typical experimental series. Bars, SE. * $= P < 0{\cdot}05$ compared with control; ** $= P < 0{\cdot}01$ compared with control.

TABLE 1. Effects of OAG and PMA on the epidermal adenylate cyclase system

	Cyclic AMP pmoles/mg protein			
	Control	OAG	PMA	Both
(a)				
Forskolin	35·7±2·4	73·7±4·8**	53·9±4·4*	74·6±6·3**
Cholera toxin	49·0±6·0	80·4±10·0*	59·8±7·6	88·0±7·1**
(b)				
Epinephrine	9·5±0·8	3·6±1·0**	5·6±0·6*	2·6±0·5**
Histamine	29·0±3·4	12·7±2·9*	19·5±1·7*	10·6±1·0**

(a) Pig epidermal sheets were incubated with 150 µg/ml OAG, 200 ng/ml PMA, or both compounds in the presence of 200 µM forskolin or 100 µg/ml cholera toxin. 1 mM IBMX was added to the incubation medium.

(b) Pig epidermal sheets were incubated with the above-mentioned chemicals for 7 h. Following incubation, epinephrine- and histamine-induced cyclic AMP accumulations were measured as described in the text.

Previously it was shown that this concentration of PMA is the maximally effective dose.[12] All $n = 4$ from a typical experimental series. $^{*} = P < 0·05$ compared with control; $^{**} = P < 0·01$ compared with control. Data are the means ± SE.

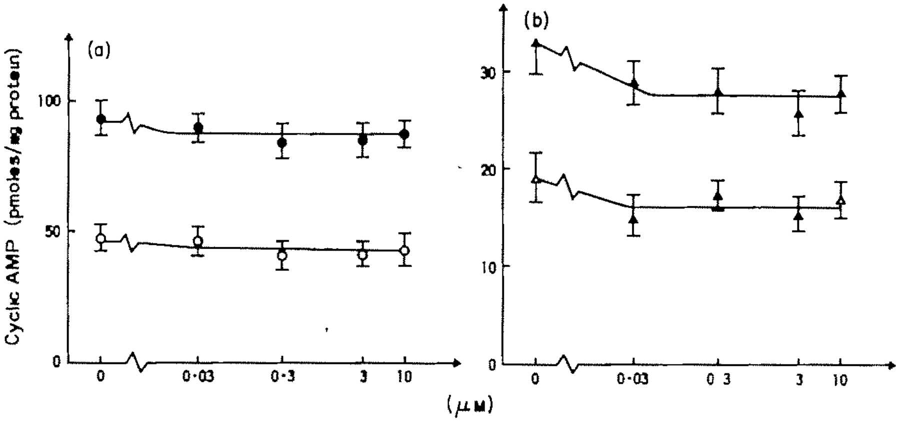

FIGURE 3. Effects of A-23187 on the epidermal adenylate cyclase responses. Pig epidermal sheets were incubated with various concentrations of A-23187. Other conditions were the same as in Figure 1. (a) Forskolin (○——○)-, and cholera toxin (●——●)-induced cyclic AMP accumulations; (b) epinephrine (△——△)-, and histamine (▲——▲)-induced cyclic AMP accumulations. All $n = 4$ from a typical experimental series. Bars, SE.

TABLE 2. Effects of A-23187 on OAG-induced modulation of epidermal adenylate cyclase system

	Cyclic AMP pmoles/mg protein			
	Control	OAG	A-23187	Both
(a)				
Forskolin	30·7 ± 2·4	52·8 ± 2·8**	27·8 ± 1·7	53·0 ± 4·0**
Cholera toxin	65·1 ± 4·3	92·6 ± 5·4**	71·6 ± 5·3	104·8 ± 9·9*
(b)				
Epinephrine	18·7 ± 1·7	9·8 ± 0·9**	15·0 ± 1·4	11·3 ± 0·6**
Histamine	40·4 ± 3·6	26·9 ± 2·6*	43·4 ± 6·6	30·0 ± 2·0*

Concentrations of chemicals were 150 μg/ml OAG, and 5 μM A-23187. Other experimental conditions were the same as in Table 1. All $n = 4$ from a typical experimental series.

* $= P < 0{\cdot}05$ compared with control; ** $= P < 0{\cdot}01$ compared with control. Data are the means ± SE.

cyclase responses (Table 2). Chelation of extracellular Ca^{2+} by EGTA slightly decreased the forskolin- and epinephrine-induced cyclic AMP accumulation (Fig. 4). However, the action of OAG was again unaffected by the EGTA; increased forskolin (as well as cholera toxin) effects and decreased receptor adenylate cyclase responses were observed following treatment with OAG and even with a markedly decreased extracellular Ca^{2+} concentration (Fig. 4).

It has been reported that by combining EGTA and A-23187, intracellular Ca^{2+} can be deleted.[22] OAG appeared to have little or no effect on the receptor responses in the presence of both A-23187 and EGTA (Fig. 5). This might suggest a toxic effect of the combinations of these agents. However, an increase of the forskolin-induced cyclic AMP accumulation was observed

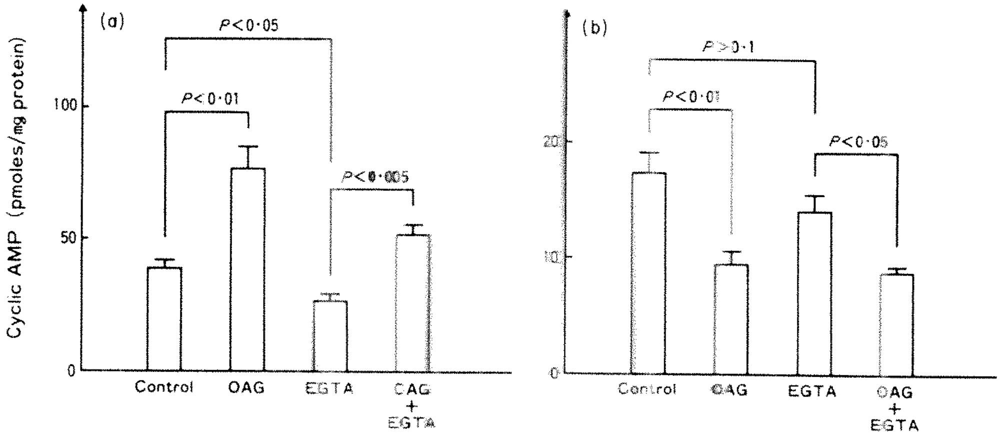

FIGURE 4. Effects of EGTA on OAG-induced adenylate cyclase modulation. Pig epidermal sheets were incubated with 150 μg/ml OAG, 5 mM EGTA, or both compounds. Extracellular Ca^{2+} concentration is estimated to be 5 nM by 5 mM EGTA in RPMI 1640 medium condition.[32] (a) Forskolin-induced cyclic AMP accumulations; (b) Epinephrine-induced cyclic AMP accumulations. All $n = 4$ from a typical experimental series. Bars, SE.

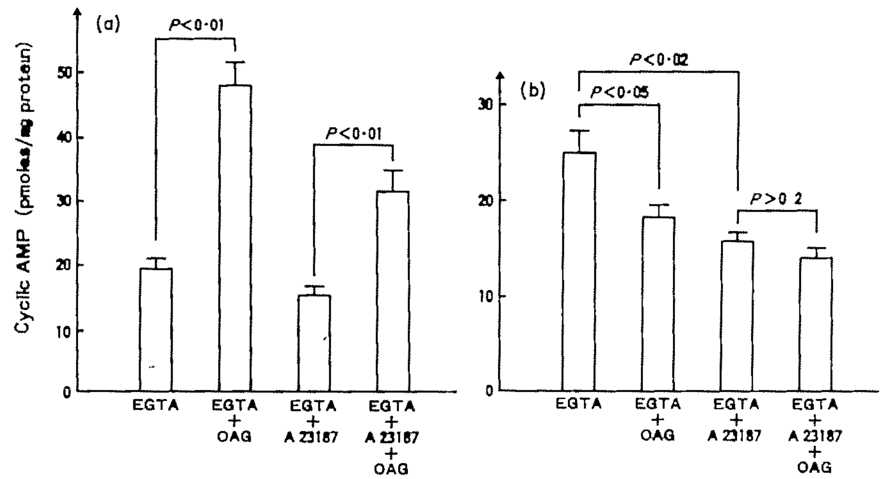

FIGURE 5. Effects of combinations of EGTA and A-23187 on OAG-induced adenylate cyclase modulation. Pig epidermal sheets were incubated with 150 μg/ml OAG, 5 mM EGTA, 3 μM A-23187, or various combinations of these compounds. Other conditions were the same as in Figure 4. (a) Forskolin-induced cyclic AMP accumulations; (b) epinephrine-induced cyclic AMP accumulations. All $n = 4$ from a typical experimental series. Bars, SE.

even in the presence of A-23187 and EGTA (Fig. 5). Thus, no evidence was obtained which suggests that modulation of Ca^{2+} results in a significant alteration of the epidermal adenylate cyclase responses.

DISCUSSION

The results indicate that OAG modulates the epidermal adenylate cyclase responses resulting in increased forskolin- and cholera toxin-induced cyclic AMP accumulation and decreased receptor–adenylate cyclase responses. The effects of OAG were similar to those of PMA,[12] although the former was generally more potent than the latter, with for example, only slight augmentation of the effect of cholera toxin by PMA. Since the effects of OAG were inhibited by the PKC inhibitor, H-7, and since the addition of both OAG and PMA to the incubation medium resulted in neither an additive nor a synergistic effect these agents appear to work at the same site, i.e. PKC.

The mechanism by which PKC modulates the epidermal adenylate cyclase responses is unknown. The decrease of receptor responses required a longer incubation time than the increase of forskolin- and cholera toxin-induced cyclic AMP accumulation and both effects were observed at a similar concentration range of OAG. Following the activation of PKC, the enzyme activity is decreased.[1] Our data indicate that modulation of the epidermal adenylate cyclase persists after 7 h incubation with OAG, probably in the presence of down-regulated PKC. Since the increased forskolin- and cholera toxin-induced cyclic AMP accumulation was observed during the first 1–3 h of incubation, it seems that the uncoupling process of R–G interaction (induced by PKC activation) is associated with the increased G–C and C functions of adenylate cyclase. We have evidence that another uncoupling process, receptor specific-refractoriness,[23] is also associated with the increased G–C and C functions.

In contrast to the PKC activators, no evidence for Ca^{2+}-induced modulation of the epidermal adenylate cyclase responses was obtained. Neither A-23187, nor EGTA (singly or in combination) showed a significant modulation of the epidermal adenylate cyclase responses. The apparent lack of effect of OAG on the receptor responses in the presence of A-23187 and EGTA might suggest a toxic effect of the combination of these agents. However, the increase of the forskolin-induced cyclic AMP accumulation was still observed in the presence of A-23187 and EGTA. Although no direct measurement of intracellular Ca^{2+} concentration was performed in the present study, the conditions used were similar to those reported to modify the intracellular Ca^{2+} in keratinocytes *in vitro*.[24–26] Topical application of A-23187 on mouse skin *in vivo* was reported to decrease the epidermal β-adrenergic adenylate cyclase response.[27] No explanation can be put forward for the discrepancy in the results from *in vivo* and *in vitro* conditions, but other systemic factors induced by the application of A-23187 might be involved *in vivo*.

It has been suggested that PI breakdown, which is the primary event of DAG and IP3 release, is related to keratinocyte proliferation[28] and differentiation.[29] The results indicate that of the two signalling systems triggered by the PI breakdown, only the DAG-PKC signalling system modulates the epidermal adenylate cyclase system.

PI breakdown is known to be modulated as a result of pathological conditions of keratinocytes. Psoriatic hyperproliferative epidermis, for example, shows increased PLC activity as well as an increased DAG content.[11,30] It is interesting to note that the psoriatic epidermis reveals very similar adenylate cyclase responses to that induced by OAG. These include increased forskolin- and cholera toxin-induced cyclic AMP accumulations[9] as well as the decreased β-adrenergic adenylate cyclase response, which is the major receptor–adenylate cyclase system of the human epidermis.[8] Also, in accordance with the down-regulated state of PKC following its activation,[31] decreased PKC activity has been found in psoriatic epidermis.[10] Thus, the altered adenylate cyclase responses observed in the epidermis in psoriasis may be due to the activation of PKC which is triggered by PI breakdown. The IP3-IP4-Ca^{2+} signalling system, which is another signalling system that is triggered by PI breakdown, appears to be irrelevant to the modulation of the epidermal adenylate cyclase responses.

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Markers for proliferation and keratinization in the margin of the active psoriatic lesion

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SUMMARY

To study the development of the psoriatic lesion, biopsies were taken from the margin of spreading plaques and acute pinpoint papules. Consecutive sections across the margin were stained using different monoclonal antibodies to characterize epidermal growth (Ki-67) and abnormal keratinization (K_s8.12, RKSE60). All three immunohistochemical markers showed pronounced changes in the lesional skin with a clear transition to the uninvolved skin. The suprabasal K_s8.12 binding was the earliest change found in the epidermis, and its localization high in the suprabasal compartment indicates that metabolic dysregulation in this cell population was not a consequence of the recruitment process in the basal layer.

Epidermal hyperproliferation, abnormal keratinization and inflammation are well-established features of the psoriatic lesion. There is growing evidence that hyperproliferation of the epidermis in psoriasis is due to entry of quiescent (G_0) cells into the cell cycle.[1,2] The majority of cycling cells can be identified by nuclear binding of the monoclonal antibody Ki-67,[3] and an increase in Ki-67$^+$ nuclei has been shown in the psoriatic lesion.[4] The suprabasal cell layers also express markers linked to increased epidermal growth, such as keratin 16, which is demonstrated by the monoclonal antibody K_s8.12.[5,6] Although this antibody also recognizes keratin 13, psoriatic epidermis does not express this keratin.[6] In psoriasis, the clinically involved skin shows a marked staining of K_s8.12, compared with uninvolved skin. The abnormal keratinization in psoriasis is characterized by the presence of nucleated corneocytes and an absence of a granular layer. Keratin 10, which is involved in the normal keratinization process and can be visualized by the monoclonal antibody RKSE60,[7] is decreased in psoriasis.[8,9] This study investigated the position of these immunohistochemical markers for hyperproliferation (Ki-67) and keratinization (K_s8.12, RKSE60) during the evolution of the psoriatic lesion.

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METHODS

Patients and biopsy procedures

Ten patients (5 male, 5 female; aged 30–56 years) with chronic plaque psoriasis participated in this study. All patients were in an unstable phase of their disease, with a short history of new and extending lesions. None of the patients received treatment for at least 2 weeks prior to the study.

In five patients a biopsy was taken with a keratotome across the transition zone of a spreading psoriatic plaque containing both uninvolved and involved skin. After cooling the skin surface with an ethyl chloride spray, a slice of 3 × 16 mm was cut using a Castroviejo keratotome (depth 0·4 mm). In five patients a razor blade biopsy (diameter 4 mm; thickness 0·5 mm) was taken from an acute pinpoint papule. Care was taken to ensure that the biopsy contained the whole papule with an annular zone of uninvolved skin.

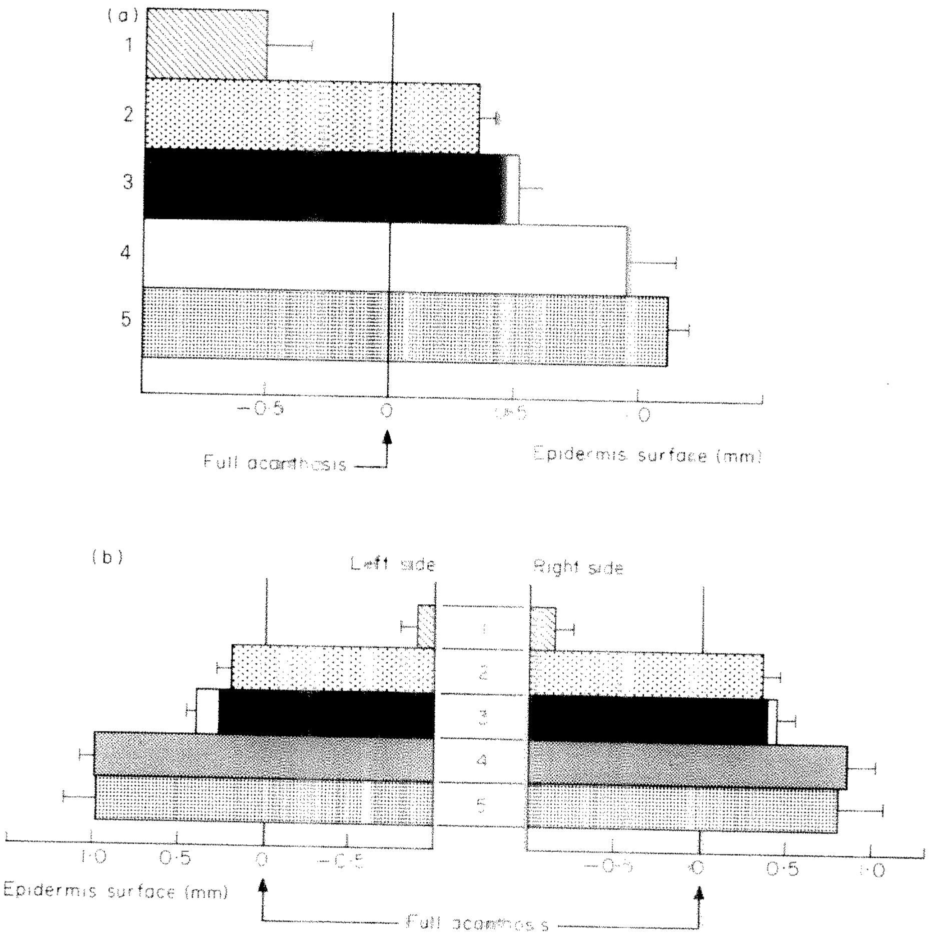

FIGURE 1. The appearance of (1) parakeratosis, (2) increased Ki-67 nuclear staining, (3) absolute and relative $K_s8.12$ staining, (4) initial acanthosis, and (5) infiltrate in mm epidermis surface (mean ± SEM) related to the appearance of full acanthosis in the margin zone of spreading psoriatic plaques (a) and acute pinpoint papules (b).

Immunohistochemical staining procedure

All biopsies were washed in phosphate-buffered saline (PBS) and frozen immediately at −20°C in Tissue Tek II OCT compound (Miles Scientific, Maperville, U.S.A.). Three series of four consecutive sections of 6 μm were cut per biopsy. In each series, one section was fixed in 70% ethanol for 1 min and stained with Hemacolor (Merck, Darmstadt, F.R.G.). The remaining three sections were fixed in acetone-ether (60/40 v/v) for 10 min and incubated with one of the following monoclonal antibodies: (1) $K_s8.12$ (Sigma, St Louis, MO, U.S.A.) diluted 1:40 in PBS, (2) RKSE60 (a gift from Dr F.C.S.Ramaekers) diluted 1 : 10 in PBS, (3) Ki-67 (Dakopatts,

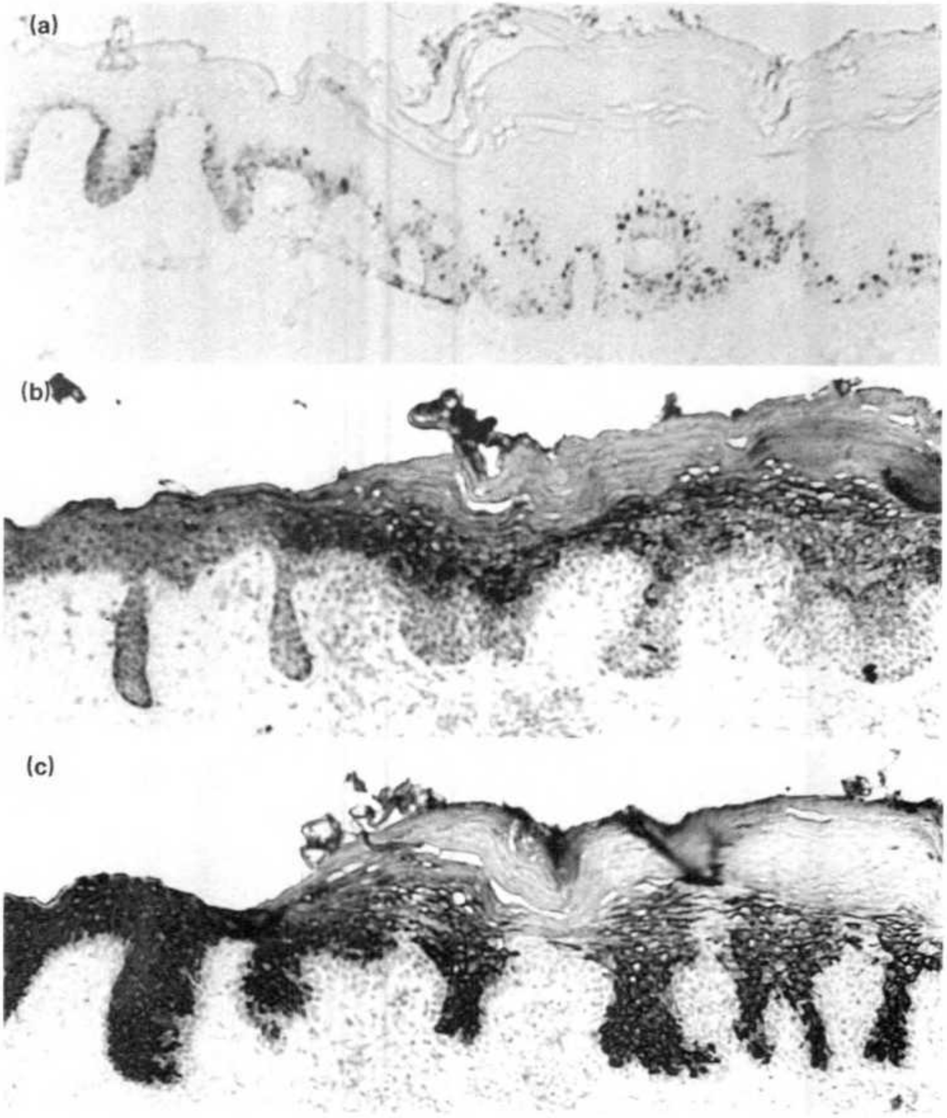

FIGURE 2. Histology of a pinpoint papule after staining with (a) Ki-67, (b) $K_s8.12$, and (c) RKSE60 (× 100).

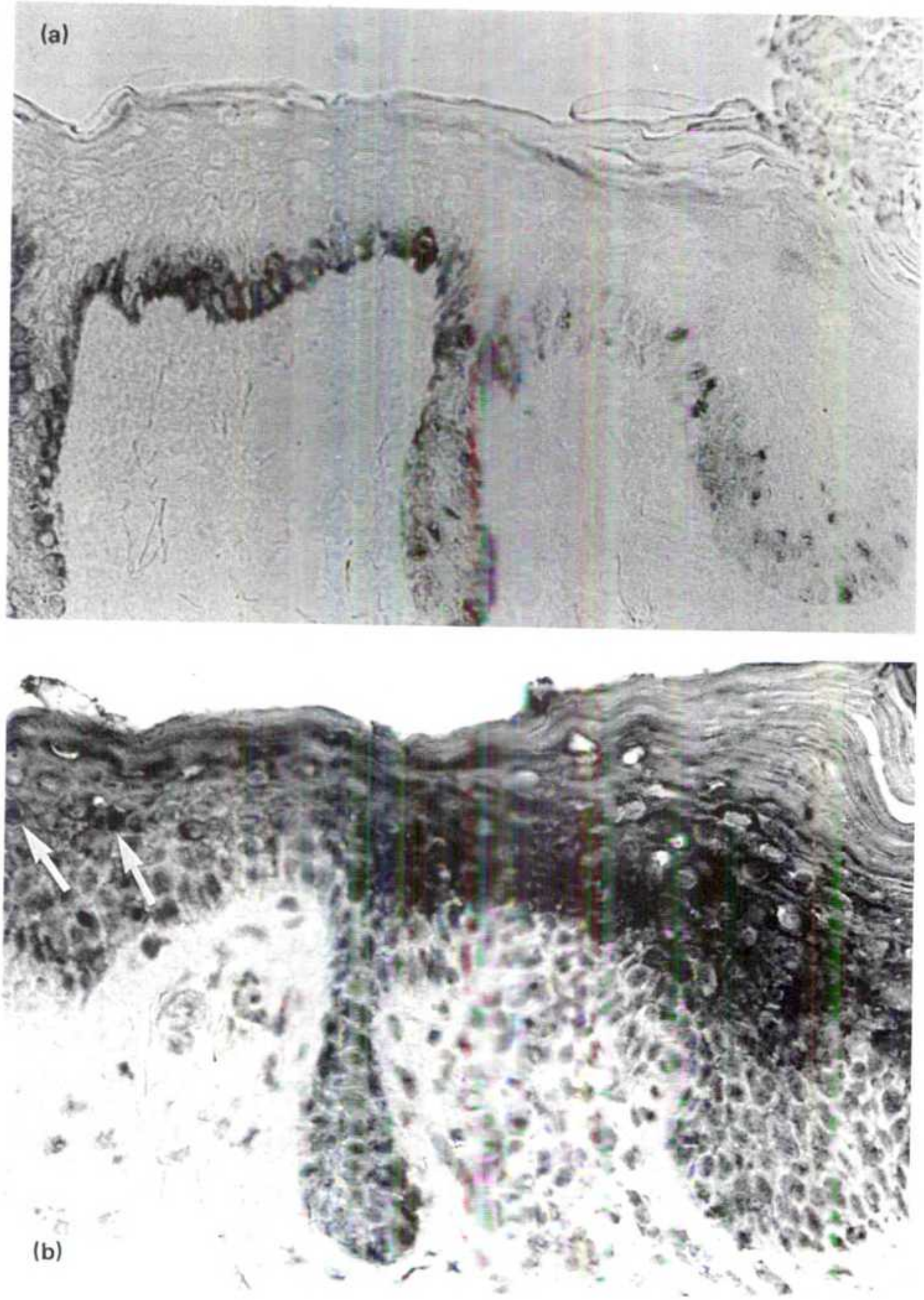

FIGURE 3. Details of the left margin of the pinpoint papule shown in Figure 2 after staining with (a) Ki-67, and (b) K$_s$8.12 (× 400). Arrows in Figure 3b indicate patches of K$_s$8.12 staining high in the suprabasal compartment.

Copenhagen, Denmark) diluted 1:20 in PBS. The bound antibodies were visualized with a standard immunoperoxidase technique as previously described.[10,11] After two washes with demineralized water, sections incubated with K_s8.12 or RKSE60 were counterstained with haematoxylin (Mayer). All sections were mounted in glycerine gelatine and studied with a Leitz 12 light microscope.

RESULTS

The classical histological features of psoriasis appeared in a consistent sequence in the marginal zone of the spreading plaques (Fig. 1a). The predominantly mononuclear infiltrate was the most peripheral feature in four out of five biopsies. The development of acanthosis reached an intermediate position, and parakeratosis had appeared after marked acanthosis had been reached in all biopsies. The topographic relationship between these histological features in pinpoint lesions is shown in Figure 1b. It can be seen that in all pinpoint lesions the infiltrate preceded the full acanthosis. However, initial acanthosis was more widespread than the infiltrate in six out of 10 of the pinpoint papules. Again, the parakeratosis was less widespread than the other histological features.

The Ki-67 staining in the margin zone of plaques and pinpoint papules showed a consistent well-defined change from cytoplasmic and sporadic nuclear staining in the uninvolved skin to exclusively nuclear staining in the lesional skin (Figs 2a and 3a). The number of Ki-67$^+$ nuclei was markedly increased in the lesion. In four out of five biopsies of the margin zone of the plaques and in eight biopsies of the pinpoint papules, the transition point of Ki-67 was localized peripherally to full acanthosis but centrally to initial acanthosis (Fig. 1).

Suprabasal binding of K_s8.12 was observed only in the psoriatic lesion and changed gradually to patchy staining of the basal layers in normal looking skin (Fig. 2b). At the periphery of the suprabasal staining, there was no continuation between staining in the higher zones of the suprabasal compartment and the patchy basal staining (Fig. 3b). Comparison of K_s8.12 binding with acanthosis is shown in Figure 1. Full suprabasal binding of K_s8.12 occurred between initial and full acanthosis in all biopsies except in one case where it preceded the initial acanthosis. In three out of five biopsies of the margin zone of the plaque and in three biopsies of the pinpoint papules, both the initial and fully expressed K_s8.12 binding coincided with the Ki-67 transition point. The remaining biopsies showed binding of K_s8.12 occurring peripherally to the Ki-67 transition point, with a marked separation between the initial and fully developed K_s8.12 binding (Fig. 1).

Comparing binding of RKSE60 and K_s8.12, a reversed staining pattern was seen with a minimal overlap between both staining patterns. RKSE60 binding was absent in the suprapapillary epidermis of the lesions, but the deeper regions of the rete ridges showed a normal RKSE60 binding with a gradually diminishing intensity towards the stratum corneum (Fig. 2c). This staining pattern was found only in the centre of the lesion.

DISCUSSION

The margins of spreading plaques and early lesions such as pinpoint papules of psoriasis allow the study of different phases of development from perilesional to lesional skin. In this model, the more peripheral changes are likely to play an early role in the pathogenesis. This study showed that the inflammatory infiltrate usually preceded the appearance of overt acanthosis, although in some cases these coincided. This observation agrees with several previous studies of pinpoint lesions[12] and the marginal zone of spreading plaques.[13] One study[14] reported that morphological changes in the epidermis preceded the dermal changes, but details as to the nature and activity of the lesions were not reported.

In the margin zone of the lesions, the monoclonal antibody Ki-67 showed a sharp transition point between 'uninvolved' and 'involved' psoriatic skin from a non-specific cytoplasmic staining to a pronounced nuclear staining representing cycling cells. If psoriatic hyperproliferation is due to recruitment of resting basal cells, the change in staining pattern of Ki-67 might be expected before the development of acanthosis. However, the Ki-67 transition point was found between initial and full acanthosis, which may be explained by a latency phase between the actual recruitment and the expression of the nuclear antigen for Ki-67. Such a timelag has been demonstrated for lymphocytes.[15]

The relationship between epidermal hyperproliferation and abnormal keratinization is still unresolved. An earlier biochemical study showed that enzyme markers for proliferation and keratinization changed simultaneously in the margin zone of the spreading lesion.[13] In this study, keratin 16 expression appeared as an early event in the margin of lesions before full acanthosis and even (in some lesions) before or coinciding with the initial acanthosis. This is in line with the early expression of keratin 16 following standardized injury.[7] The isolated localization of the initial keratin 16 expression high in the suprabasal compartment implies that these biochemical changes are not a consequence of metabolic events associated with the recruitment of G_0-cells in the basal cell layer. We suggest that the changes in the suprabasal compartment are a link between the induction of regeneration and the recruitment process in the basal layer. Alternatively, both processes might be the consequence of a signal which is transduced to the basal and suprabasal compartment at different times.

The reverse staining pattern of RKSE60 compared to $K_s8.12$ indicates the possibility of a down regulation of keratin 10 caused by the induction of keratin 16 expression. Such an inverse relationship between keratin 10 and keratin 16 has also been shown in other skin diseases[8] and regenerative epidermis.[6,16] However, this study demonstrates an interphase of simultaneous expression of both keratins 10 and 16, suggesting that both normal and hyperproliferative maturation pathways can exist together. Alternatively, the rate of disappearance of epitopes recognized by RKSE60 might be of relevance in this respect.

As the infiltrate was seen in the most peripheral zones of the spreading plaques and pinpoint papules, analysis of these cells using immunohistochemical markers might further our understanding of the pathogenesis of psoriasis. Of all the immunohistochemical parameters investigated in this study, the binding of $K_s8.12$ in the suprabasal compartment represented the earliest event in the development of epidermal changes.

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Beta-adrenergic stimulation of cyclic AMP is defective in cultured dermal fibroblasts of psoriatic subjects

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SUMMARY

Epidermal cells from psoriatic lesions demonstrate a very low cAMP response to beta-adrenergic stimuli. We have shown that a similar abnormality occurs in dermal fibroblasts from affected areas of skin. The cells, after 5–12 passages in tissue culture, had a much reduced response to 10^{-8} M and 10^{-6} M isoproterenol when compared with fibroblasts from control subjects. The abnormality was not abolished by the addition of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine. Other putative agonists tested were vasoactive intestinal peptide and peptide histidine methionine. Neither of these had an effect on dermal fibroblasts from either normal controls or from lesions of psoriasis.

Psoriasis is characterized by epidermal hyperproliferation and reduced epidermal cell differentiation. Recent studies have shown that the earliest histological changes occur within the dermis and include dilatation of capillaries[1] and accumulation of leucocytes.[2] Furthermore, the grafting of psoriatic skin into athymic mice[3] and Köbner experiments have demonstrated an interaction between the dermis and epidermis during the development of the lesions in psoriasis.[4] It is believed that the changes in the dermis are essential for the subsequent development of the epidermal lesions.[5]

A number of biochemical abnormalities have been found in the dermis of affected psoriatic skin which include an increased amount of glycosaminoglycans associated with the fibroblasts.[6] When studied *in vitro*, fibroblasts from affected psoriatic skin exhibit an increased rate of proliferation compared to those from control tissue and in this respect resemble the epidermal cells.[7]

Cyclic AMP (cAMP) has been implicated as a modulator of cellular growth and differentiation in a variety of cell types.[8–10] In psoriasis, an increased rate of proliferation has been

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associated with a specific reduction in beta-adrenergic responsiveness in epidermal cells.[11,12] This study investigated whether the same abnormality occurred in dermal fibroblasts in psoriasis. We also investigated whether vasoactive intestinal peptide (VIP) and peptide histidine methionine (PHM), both found in unmyelinated nerve fibres in human skin,[13–15] could modulate cAMP levels in the dermal fibroblasts.

METHODS

Control subjects

Redundant abdominal skin was obtained from patients (three female and one male, age range 48–69 years) undergoing elective surgery. None of the patients had any history of skin disease.

Psoriatic patients

Punch biopsies of lesional skin were obtained from two female and two male patients with psoriasis (age range 35–44 years), under local anaesthesia. All had extensive psoriasis and were treated with topical emollients alone.

Establishment of primary cell cultures

Fibroblast cell lines were established by the primary explant technique. The cultures were maintained in tissue culture flasks (Becton Dickenson Co, Oxnard, California, U.S.A.) with RPMI 1640 medium (Gibco, Paisley, Scotland) containing 10% fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin. The flasks were exposed to an atmosphere of 95% air and 5% CO_2 in an incubator at 37°C. The culture medium was replaced twice weekly and fibroblasts harvested by trypsinization when a confluent monolayer had been achieved. Seeding was performed on to a growth surface no greater than three times that of the confluent monolayer of cells. Cells were seeded into 35 mm diameter Petri dishes (Becton Dickenson Co, Oxnard, California, U.S.A.). Cells were only used after reaching confluence between passage 5 and 12 and at least 7 days from the last trypsinization.

Incubation of fibroblasts

Solutions containing isoproterenol, VIP, PHM and the phosphodiesterase inhibitor 3 isobutyl-1-methylxanthine (IBMX) were prepared at 100-fold final concentrations in RPMI containing 0·1% albumin and kept at 4°C prior to use. Before incubation fibroblasts were washed twice with fresh RPMI. Then 1·5 ml fresh RPMI containing 0·1% albumin was added and the cells left to equilibrate for 15 min at 37°C. At this point, 15 μl of agonist and either 15 μl of IBMX or 15 μl of RPMI containing 0·1% albumin were added to the Petri dishes and left for 5 min at 37°C. For basal conditions 30 μl RPMI 0·1% albumin were added. Incubations were usually performed in triplicate (occasionally duplicate or quadruplicate) and were stopped by the addition of 1·5 ml 20% (w/v) ice-cold trichloroacetic acid. The Petri dishes were then placed on ice for 15 min. The cellular protein was dislodged from the Petri using a rubber policeman. The whole Petri contents were centrifuged at 1500 *g* for 15 min and 1 ml supernatant saved for cAMP estimation. The pellet was kept for protein measurement.

cAMP extractions and estimations

Trichloroacetic acid was extracted from the supernatant solution using 4 × 5 ml volumes of water-saturated diethyl ether. The aqueous phase was dried in a stream of air and cAMP measured by means of radioimmunoassay (Amersham International plc, Amersham, Bucks, U.K.).

Protein estimations

Proteins from the cell pellets were solubilized using 0·5 ml of 1 M NaOH and the protein assayed by the method of Bradford,[16] using a Biorad protein assay kit (BioRad Labs GmbH, Munchen, West Germany).

VIP and PHM radioimmunoassay

In these experiments, both VIP and PHM were added at 10^{-6} M concentrations. However, since both VIP and PHM are known to adhere to glass and plastic, the concentrations remaining in solution under the identical conditions used in the experiments were measured using radioimmunoassays previously described.[17,18] Under these conditions 70–90% of the calculated amount of peptide was present at the end of the experiment.

Statistics

The results were calculated as pmol cAMP/mg protein and expressed as means ± standard error. Statistical analysis was performed using the unpaired Student's t-test.

RESULTS

The cyclic AMP levels measured following incubation of the fibroblasts with putative agonists are shown in Table 1. The basal cyclic AMP levels were significantly lower in the psoriatic than in the control fibroblasts, both in the absence ($P<0\cdot001$) and in the presence of IBMX ($P<0\cdot05$). Stimulation by 10^{-8} M isoproterenol was significantly less in the psoriatic fibroblasts than in the controls. Stimulation by 10^{-6} M isoproterenol both in the absence and presence of

TABLE 1. cAMP levels in cultured dermal fibroblasts from normal and psoriatic lesional skin

Agonist	IBMX (10^{-4} M)	cAMP (pmol/mg protein) Control	cAMP (pmol/mg protein) Psoriasis
—	–	7·1 ± 0·6	4·5 ± 0·2**
—	+	7·2 ± 0·8	4·8 ± 0·4*
ISO 10^{-8} M	–	84·2 ± 1·2	7·4 ± 0·8**
ISO 10^{-6} M	–	200·0 ± 37·7	41·0 ± 5·7**
ISO 10^{-6} M	+	267·0 ± 44·6	47·1 ± 7·8**
VIP 10^{-6} M	–	6·5 ± 0·9	3·7 ± 1·3
VIP 10^{-6} M	+	5·8 ± 0·2	6·5 ± 1·0
PHM 10^{-6} M	–	5·3 ± 0·6	4·3 ± 0·8
PHM 10^{-6} M	+	7·2 ± 0·8	6·8 ± 1·6

* $P<0\cdot05$ } vs. control fibroblasts.
** $P<0\cdot001$ }

Dermal fibroblasts were grown to confluence in Petri dishes. The results were obtained from three to four independent experiments for each stimulant and the number of replicate dishes for each condition per experiment was usually three (range two to four).

IBMX was significantly less in the psoriatic fibroblasts than in the controls ($P < 0{\cdot}001$), the addition of IBMX 10^{-4} M having little effect on cAMP levels in psoriatic fibroblasts.

Incubations with 10^{-6} M VIP and 10^{-6} M PHM, both in the absence and presence of IBMX, failed to increase cAMP levels significantly in either the control or psoriatic fibroblasts.

DISCUSSION

These results clearly demonstrate that in psoriasis the response of dermal fibroblasts derived from lesional skin to beta-adrenergic stimulation by isoproterenol is much reduced compared to fibroblasts derived from normal control skin.

The basal cAMP levels measured in normal fibroblasts which had reached confluency, were of the same order of magnitude as that found in other studies.[19,20] Stimulation of normal fibroblast cultures with 10^{-6} M isoproterenol produced a 28-fold increase in the cAMP accumulation, with a further increase to 34-fold with the addition of the phosphodiesterase inhibitor, IBMX. Stimulation with 10^{-8} M isoproterenol gave a 10-fold increase over the basal levels. It was found that the basal cAMP levels in psoriatic fibroblasts were significantly less than those for controls. The increase in cAMP in psoriatic fibroblasts with 10^{-8} M and 10^{-6} M isoproterenol was 1·6- and ninefold, respectively. Both in the absence of isoproterenol and in the presence of 10^{-6} M isoproterenol, the addition of the phosphodiesterase inhibitor, IBMX, had little effect on cAMP levels in psoriatic fibroblasts. This indicates that the lower cAMP levels found in psoriatic compared with control fibroblasts were unlikely to be caused by increased phosphodiesterase activity in the former. Since all our experiments were carried out following 5 min of stimulation with agonist, we cannot rule out that psoriatic fibroblasts achieved maximal levels at a later time than did controls, but we think this unlikely. Another reason for the differences in cAMP levels between normal and psoriatic fibroblasts could be that the increased macromolecule secretion[6] or the poorer cell adhesive properties of psoriatic fibroblasts (to plastic)[7] may alter cyclic nucleotide metabolism. Our present experiments do not allow us to evaluate this possibility.

Our results suggest that dermal fibroblasts may have the same abnormality of beta-adrenergic transmission as that described for epidermal cells in psoriasis.[11,12,21,22] Work on the unstimulated cAMP content of involved psoriatic epidermis is conflicting, being reported as lower,[23,24] higher[25] or the same[26–29] as normal controls. These discrepancies may be due to differences in the age of the clinical lesions sampled, the tissue sampling techniques used, and the ischaemic time of the keratotomed epidermis.[30] Aoyagi *et al.*[31] found that the cAMP levels were reduced in the basal proliferative compartment of the epidermis, while the total epidermis had higher levels of cAMP overall. Interpretation is made more difficult by the finding of increased activity of cAMP-dependent phosphodiesterases in the lower half of the epidermis.[32] These findings are also supported by the clinical observation that drugs such as lithium[33,34] and propranolol,[35] which are known to inhibit cAMP production, cause exacerbation of psoriasis, while topically applied isoproterenol results in clinical improvement.[36] Deficiencies of the cAMP-dependent protein kinases have been reported in both the plasma membranes of circulating erythrocytes of patients with psoriasis and in psoriatic fibroblasts.[37] This effect can be reversed by incubating with etretinate, a drug used in the treatment of psoriasis.[38] Our results show that dermal fibroblasts from psoriatic tissue have an abnormality of beta-adrenergic transmission not previously described for these cells, which occurs in addition to the defect in cAMP-dependent protein kinases already reported.[38] Furthermore, since the results are from cultured fibroblasts which have undergone five or more passages in cell culture, the abnormality cannot be due to circulating factors.

Rosengurt[39] suggested from his work on quiescent serum-starved 3T3 fibroblasts that regulatory peptides and other polypeptide growth factors may be important extracellular signals initiating intracellular secondary messenger control over DNA synthesis and regulation of cell proliferation, growth and differentiation. It has been speculated that the release of neuropeptides from unmyelinated nerve endings in the skin may play a role in the pathogenesis of psoriasis.[40] The exacerbation of psoriasis by stress and the symmetry of lesions of psoriasis suggest that a neurogenic agent such as neuropeptides may be implicated. In this study, the effect of neuropeptides VIP and PHM on cAMP generation in cultured fibroblasts from normal and psoriatic individuals was investigated. These neuropeptides did not stimulate cAMP generation in cultured human fibroblasts either in the presence or absence of a phosphodiesterase inhibitor. VIP stimulates adenylate cyclase activity in a wide variety of tissues, including fat and liver cell membranes,[41,42] pancreatic acini,[43,44] and intestinal mucosa[45] and in the skin stimulates eccrine sweat glands.[46] Since they did not generate any cAMP in dermal fibroblasts it seems unlikely that either VIP or PHM act on the human fibroblast, at least by this mechanisms.

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Serum levels of trace metals in children with atopic eczema

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SUMMARY

The hypothesis that atopic eczema is associated with a non-specific decrease in the serum concentration of trace metals was examined by measurement of the levels of zinc, copper and iron in 134 children with atopic eczema and 112 controls. The results failed to confirm the hypothesis. There was no significant difference between patients and controls for the concentrations of serum zinc, iron, iron-binding capacity and albumin. Patients with eczema had a significantly higher copper concentration (median 21·0 μmol/l) than controls (median 17·0 μmol/l), and a significantly lower concentration of ferritin (median 11·9 ng/ml for patients and 16·5 ng/ml for controls). There was a highly significant correlation between the surface area of skin affected by eczema and the concentrations of albumin and orosomucoid.

We previously reported a significantly reduced serum zinc concentration in 65 children with atopic eczema.[1] There was no correlation between the serum zinc concentration and either the height/weight centile or a subjective assessment of the severity and extent of the eczema. We suggested that the decreased serum zinc concentration was a non-specific consequence of the dermatological disorder, analogous to a low serum iron concentration as found in various dermatoses.[2] To test this hypothesis we studied a further sample of patients, and extended the study to two other trace metals, copper and iron.

METHODS

One-hundred and thirty-four children with atopic eczema were studied. The patients were not on systemic corticosteroids. The surface area affected by eczema was assessed, and the severity of the erythema was scored by TJD as mild (1), moderate (2) or severe (3). A combined score, where the surface area was multiplied by the severity score, was calculated. Venepuncture was

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carried out between 11·00 and 12·00 hours because of the effects of diurnal variation of the serum zinc, copper and iron concentrations.[3–5] For copper and zinc the blood was collected into plain plastic tubes and the serum separated before measurement by conventional atomic absorption spectrophotometry[5] (Pye SP 192). Serum iron was measured by direct aspiration of 75 μl aliquots of undiluted serum to the flame of an atomic absorption spectrophotometer equipped with a peak-height integrator[6,7] (Pye SP 199). For measurement of iron-binding capacity (IBC) serum was saturated with iron and the excess removed by absorption onto magnesium carbonate.[8] The concentration of iron in the treated serum was measured as described above, with care being taken to avoid haemolysis. Orosomucoid (α-1-acid glycoprotein) was measured by electroimmunoassay,[9] and albumin was measured by a dye-binding procedure employing bromocresol purple.[10] Ferritin was measured by an ELISA test.[11] The 112 controls were children who were having blood taken for blood grouping prior to elective surgery.

RESULTS

The patients with atopic eczema had significantly higher serum copper and orosomucoid concentrations, and significantly lower serum ferritin concentrations, than the controls (Table 1). The serum concentrations of zinc, iron, albumin, and iron-binding capacity did not differ significantly between the two groups. There was a significant correlation between the surface area affected by eczema and the serum concentrations of orosomucoid and albumin, but no significant correlation with the concentrations of zinc, copper, iron, iron binding or ferritin (Table 2).

The patients as a group were significantly younger than the controls. Examining the effect of age, using Spearman's correlation coefficient, it was found that the serum concentrations of zinc and copper correlated with age in both groups, zinc rising and copper falling with increasing age (Table 3). The serum iron concentration rose significantly with increasing age in the patient group, and the ferritin concentration fell significantly with age in the control group. In order to confirm that any statistically significant differences between patients and controls were not the result of different age distributions, the regression lines of serum concentration of copper, ferritin, iron and zinc against age were calculated separately for patients and controls. The

TABLE 1. Summary of results, comparing patients and controls

	Median (n)			
Variable	Controls	Patients	*U*-value*	*P*
Age (years)	9·78 (112)	3·14 (134)	4150	< 0·0001**
Zinc (μmol/l)	11·5 (112)	11·5 (133)	6867	0·29
Copper (μmol/l)	17·0 (112)	22·0 (130)	3854	< 0·0001**
Iron (μmol/l)	13·2 (111)	12·5 (128)	6177	0·082
Iron-binding capacity (μmol/l)	59·0 (111)	60·0 (128)	6395	0·18
Orosomucoid (mg/l)	720 (111)	870 (127)	5035	0·0001**
Albumin (g/l)	36·1 (111)	36·2 (126)	6934	0·91
Ferritin (ng/ml)	16·5 (100)	11·9 (104)	3681	0·0003**

* Mann–Whitney *U*-test.

** Significant result, $P < 0.05$.

TABLE 2. Effect of surface area and severity of eczema

		Surface area		Combined score*	
Variable	n	Spearman correlation coefficient	P	Spearman correlation coefficient	P
Age	134	−0·17	0·05	−0·13	0·15
Zinc	133	−0·06	0·50	−0·03	0·70
Copper	130	0·06	0·52	0·08	0·36
Iron	128	0·17	0·06	−0·17	0·06
Iron binding	128	−0·05	0·60	−0·06	0·50
Ferritin	104	−0·12	0·22	−0·13	0·20
Orosomucoid	127	0·29	0·001**	0·29	0·001**
Albumin	126	−0·33	0·001**	−0·36	0·001**

* Surface area multiplied by severity of erythema score (see text)
** Significant correlation, $P<0\cdot05$.

slopes of the regression lines were not significantly different for any of these substances. The standard error of the vertical difference between the two lines was calculated, to see whether it was significantly different from zero by comparing $z=(d-0)/\mathrm{SE}(d)$ with a standard normal distribution, using a two-sided test, where z = standard normal deviate, d = vertical difference between the lines assuming a common slope and SE (d) = standard error of the vertical difference between the two lines. For the serum iron concentration, the slopes of the regression lines against age were significantly different for patients and controls. Taking this into account, the vertical distance between the two lines at the time point corresponding to the mean age of the patients was compared to see if it was significantly different from zero. The results (Table 4), showed that for the serum copper and ferritin there are differences between the levels for

TABLE 3. Effect of age

	Controls (n)		Patients (n)	
Variable	Spearman correlation coefficient	P	Spearman correlation coefficient	P
Surface area	—		−0·17 (134)	0·05
Combined score*	—		−0·13 (134)	0·15
Zinc	0·26 (112)	0·006**	0·37 (133)	0·001**
Copper	−0·26 (112)	0·006**	−0·25 (130)	0·006**
Iron	0·15 (111)	0·13	0·23 (128)	0·01**
Iron binding	0·12 (111)	0·22	−0·02 (128)	0·82
Orosomucoid	−0·06 (111)	0·54	−0·08 (127)	0·36
Albumin	0·12 (111)	0·22	0·14 (126)	0·13
Ferritin	−0·20 (100)	0·04**	−0·15 (104)	0·12

* Surface area multiplied by severity of erythema score (see text).
** Significant, $P<0\cdot05$.

TABLE 4. Comparison between patients and controls, taking into account the relationship of serum concentration against age (see text for details)

Substance	z	P
Zinc	−1·50	>0·1
Copper	−4·34	<0·0001
Ferritin	4·78	<0·00001
Iron	1·29	>0·1

A negative sign for z indicates that patients had higher values than controls.

patients and controls, and these are not due to differences in the age distribution of the two groups. For the serum zinc and iron the differences between the patients and controls were not significant, and this was not due to the differences in age of the two groups.

DISCUSSION

The concentration of serum zinc (median 11·5 μmol/l) in patients with atopic eczema was almost identical to the figure obtained in our previous study (median 11·3 μmol/l).[1] The concentration of serum zinc in the control group (median 11·5 μmol/l) was lower than in the control group used in the previous study (median 13·4 μmol/l), and this explains the lack of difference between patients and controls found in this study. In both series the controls tended to be older than the patients, but the differences between the control groups is unexplained. Our latest findings are consistent with the recent reports of normal levels of zinc in the blood of 21 eczematous children[12] and 24 eczematous adults.[13]

The serum copper concentration was significantly higher in children with eczema. This is consistent with the finding of an elevated level of serum copper and caeruloplasmin in 24 eczematous adults[13] but is at variance with the normal level of serum copper found in 21 eczematous children.[12] Over 90% of copper in serum is bound to caeruloplasmin,[14] an acute phase reactive protein, and it has been suggested that the increased concentrations of copper and caeruloplasmin are attributable to the inflammation associated with the skin disease.[13] This is supported by our finding of a significantly elevated level of orosomucoid, another acute phase reactant,[15] but not by the lack of correlation between serum copper and disease severity, found in this and a previous study.[13]

There was no significant difference in serum iron concentration between patients and controls, although the serum ferritin was significantly lower in the patients, possibly suggesting a depletion of body iron stores. This could be due to a poor dietary intake of iron, malabsorption associated with atopic eczema,[16] or iron loss in exfoliated skin cells.[2]

The hypothesis that atopic eczema is associated with a non-specific decrease in the serum concentration of trace metals such as zinc, copper or iron is not supported by this study. A coincidental finding was a highly significant correlation between the surface area of skin affected by eczema and the concentrations of albumin and orosomucoid.

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Dose–response relationships for topically applied antigens

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SUMMARY

Patch-test responses to serially diluted nickel sulphate and potassium dichromate solutions in patients with nickel and chrome sensitivity were assessed by pulsed A-scan ultrasound to determine skin thickness and laser-Doppler flowmeter techniques to evaluate blood flow, as well as clinical scoring. There was a relationship between the dose of antigen and the test responses as measured by ultrasound and laser-Doppler flowmeter techniques in both nickel and chrome sensitive patients. Our data suggest that both techniques may be useful in the detection of allergic contact dermatitis in which there is a doubtful patch-test reaction as well as in experimental sensitization studies.

Since the proposals of the International Contact Dermatitis Research Group in 1970,[1] a clinical rating scale for the evaluation of patch-test reactions is now generally accepted. However, the reading of patch-test reactions is still subjective, depending on the observer's eye and skill.[2]

Recently, several objective methods of evaluating patch-test and irritant reactions have been developed, including laser-Doppler flowmetry,[3–6] ultrasound,[7,8] skin evaporimetry,[9–11] skin calipers,[12] transcutaneous PO_2 measurement,[13] contact thermography[14] and skin-reflectance measurement.[15] Most have focused on the standard, clinically used patch-test concentrations when testing instrumental methods to replace clinical grading. Some authors[5,7] have suggested that differentiation between weak and strong reactions could be achieved by laser-Doppler and ultrasound methods, but they could not differentiate between negative and doubtful reactions. Other authors[8] stated that there was too much overlap between different grades of positive reaction to be of value in routine clinical use.

Serial dilution of the antigens, particularly below the standard patch-test concentration, and measurement of responses by objective instruments can give us more information about dose-response. To investigate whether there is any correlation between antigen dose and reaction produced to a patch test, we applied serially diluted antigens and measured their responses by laser-Doppler flowmetry and by ultrasound as well as clinical scoring. We also wanted to find

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out whether these objective methods correlated with each other and whether they could be useful for the determination of the end point of patch-test reactions.

METHODS

Nickel and potassium dichromate were chosen as antigens because both can be diluted easily in solution and it was relatively easy to recruit patients who were sensitized to these antigens.

Subjects

Twenty nickel sensitive and nine chrome sensitive patients, who had attended the contact dermatitis clinic were included in this study, all having given their informed consent. By the International Contact Dermatitis grading scale[1] the nickel sensitive patients consisted of four + reactors, 12 + + reactors and four + + + reactors, and the chrome sensitive patients consisted of two + reactors, four + + reactors and three + + + reactors when they were tested in our study with the highest dilutions of the antigens (5% nickel sulphate in distilled water, 0·5% potassium dichromate in distilled water).

Antigens

Nickel sulphate and potassium dichromate were serially diluted below the standard patch-test concentrations. Nickel sulphate $7\ H_2O$ (Analar®, BDH Chemicals, Poole, Dorset, U.K.) was adjusted to 5% (weight/volume) in distilled water and serially diluted by halves, i.e. $5 \times (\frac{1}{2})^1\%$, $5 \times (\frac{1}{2})^2\%$, $5 \times (\frac{1}{2})^3\%$, $5 \times (\frac{1}{2})^4\%$, $5 \times (\frac{1}{2})^5\%$, $5 \times (\frac{1}{2})^6\%$, $5 \times (\frac{1}{2})^7\%$. Potassium dichromate (BDH Chemicals) was adjusted to 0·5% (weight/volume) in distilled water and serially diluted by halves, i.e. $0{\cdot}5 \times (\frac{1}{2})^1\%$, $0{\cdot}5 \times (\frac{1}{2})^2\%$, $0{\cdot}5 \times (\frac{1}{2})^3\%$, $0{\cdot}5 \times (\frac{1}{2})^4\%$, $0{\cdot}5 \times (\frac{1}{2})^5\%$, $0{\cdot}5 \times (\frac{1}{2})^6\%$, $0{\cdot}5 \times (\frac{1}{2})^7\%$.

Patch-test application and reading

Quantities of each diluted solution (15 µl) were delivered to separate filter-paper discs, placed in Finn chambers on Scanpor tape and then applied to the skin of the upper back of the patients as in a routine patch test. Each patient was therefore challenged with seven serial dilutions of the appropriate antigen plus one blank control (distilled water). An area of normal skin served as untreated control from which relative responses were assessed.

The chambers were removed 48 h later and sites were assessed approximately 1 h and 2 days after the removal (2-day and 4-day readings). All test sites were assessed by visual reading as recommended by the International Contact Dermatitis Research Group.[1] Readings of skin thickness were taken using an ultrasound A-scan device (Cutech Dermal Monitor 70) and measurements of blood flow in the superficial dermis were made using a laser-Doppler flowmeter (Perimed; LKB Pharmacia) as previously described.[3–5]

Statistical analysis

All analyses were performed using the relative response, i.e. reading obtained at test site minus reading from the unchallenged skin. All dose-response relationships were calculated using the $\log[Ni^{2+}]\%$ w/v.

RESULTS

The responses to the different concentrations of nickel sulphate and potassium dichromate were measured by ultrasound and laser-Doppler flowmeter techniques, and expressed as absolute

TABLE 1. Dose–response according to the different concentrations of nickel sulphate by ultrasound and laser-Doppler measurements (mean ± standard deviation)

	Ultrasound (mm)		Laser-Doppler (arbitrary units)	
	No. of cases		No. of cases	
	19	19	20	19
% Concentration	2 Days	4 Days	2 Days	4 Days
5	4·53 ± 0·94	4·14 ± 0·66	0·90 ± 0·26	0·85 ± 0·33
$5 \times (\frac{1}{2})^1$	4·19 ± 0·88	4·08 ± 0·63	0·83 ± 0·30	0·86 ± 0·41
$5 \times (\frac{1}{2})^2$	3·73 ± 0·77	3·91 ± 0·83	0·77 ± 0·37	0·71 ± 0·48
$5 \times (\frac{1}{2})^3$	3·66 ± 0·71	3·78 ± 0·54	0·70 ± 0·41	0·60 ± 0·39
$5 \times (\frac{1}{2})^4$	3·59 ± 0·65	3·59 ± 0·68	0·53 ± 0·35	0·52 ± 0·45
$5 \times (\frac{1}{2})^5$	3·48 ± 0·66	3·38 ± 0·61	0·50 ± 0·35	0·39 ± 0·34
$5 \times (\frac{1}{2})^6$	3·52 ± 0·69	3·49 ± 0·63	0·44 ± 0·35	0·38 ± 0·29
$5 \times (\frac{1}{2})^7$	3·43 ± 0·59	3·43 ± 0·79	0·26 ± 0·25	0·19 ± 0·13
Distilled water	3·19 ± 0·59	3·16 ± 0·56	0·21 ± 0·17	0·11 ± 0·03
Normal skin	3·06 ± 0·56	3·02 ± 0·51	0·09 ± 0·05	0·10 ± 0·04

TABLE 2. Dose–response according to the different concentrations of potassium dichromate by ultrasound and laser-Doppler measurements (mean ± standard deviation)

	Ultrasound (mm)		Laser-Doppler (arbitrary units)	
	No. of cases		No. of cases	
	8	8	9	8
% Concentration	2 Days	4 Days	2 Days	4 Days
0·5	4·67 ± 0·80	4·52 ± 0·82	0·95 ± 0·30	0·78 ± 0·35
$0·5 \times (\frac{1}{2})^1$	4·30 ± 0·63	4·29 ± 0·59	0·83 ± 0·40	0·74 ± 0·37
$0·5 \times (\frac{1}{2})^2$	3·82 ± 0·75	3·96 ± 0·82	0·68 ± 0·41	0·70 ± 0·36
$0·5 \times (\frac{1}{2})^3$	4·02 ± 0·75	3·84 ± 0·77	0·68 ± 0·47	0·63 ± 0·35
$0·5 \times (\frac{1}{2})^4$	3·84 ± 0·55	3·95 ± 0·73	0·64 ± 0·41	0·56 ± 0·41
$0·5 \times (\frac{1}{2})^5$	3·52 ± 0·60*	3·80 ± 0·74*	0·43 ± 0·31**	0·34 ± 0·33*
$0·5 \times (\frac{1}{2})^6$	3·73 ± 0·68*	3·67 ± 0·72*	0·42 ± 0·41**	0·41 ± 0·32*
$0·5 \times (\frac{1}{2})^7$	3·55 ± 0·72*	3·61 ± 0·72*	0·19 ± 0·09**	0·18 ± 0·14*
Distilled water	3·50 ± 0·79	3·58 ± 0·76	0·13 ± 0·05	0·13 ± 0·05
Normal skin	3·26 ± 0·66	3·38 ± 0·74	0·12 ± 0·04	0·13 ± 0·03

* No. cases 7.

** No. cases 8.

values in Tables 1 and 2. These tables show that there is a relationship between antigen dose and patch-test response as measured by the two instruments. Similar responses were observed for both the 2-day and the 4-day readings. The skin thickness results were also plotted in graphic form as absolute and relative values (the relative value = absolute value − thickness value of normal skin) in the nickel-sensitive patients in Figure 1 (ultrasound) and Figure 2 (laser Doppler). They show a dose–response relationship. Correlation between antigen dose and ultrasound reading was found with both absolute and relative values at both 2 days and 4 days ($P < 0·001$). Laser-Doppler readings at 2 and 4 days also correlated positively with antigen dose ($P < 0·01$) for both absolute and relative values. Almost the same curves were observed in the potassium dichromate-sensitive patients.

The scatter diagrams of $5 \times (\frac{1}{2})^7$% nickel sulphate versus corresponding response to distilled-water occlusion measured by ultrasound is shown in Figure 3(a) and laser-Doppler flowmetry in Figure 3(b). They show that nearly all points lie above the diagonal lines of equality, which means the measurements can differentiate between allergic patch-test responses and distilled-water occlusion responses at this dilution. They also show clearly that in laser-Doppler measurement the value obtained from normal skin is negligible compared to the values of patch-test reactions. This is in contrast to ultrasound measurement where readings from normal skin can be more than half the values obtained from positive reactions. Similar scatter diagrams were observed in the potassium dichromate-sensitive patients. The scatter diagrams of the response

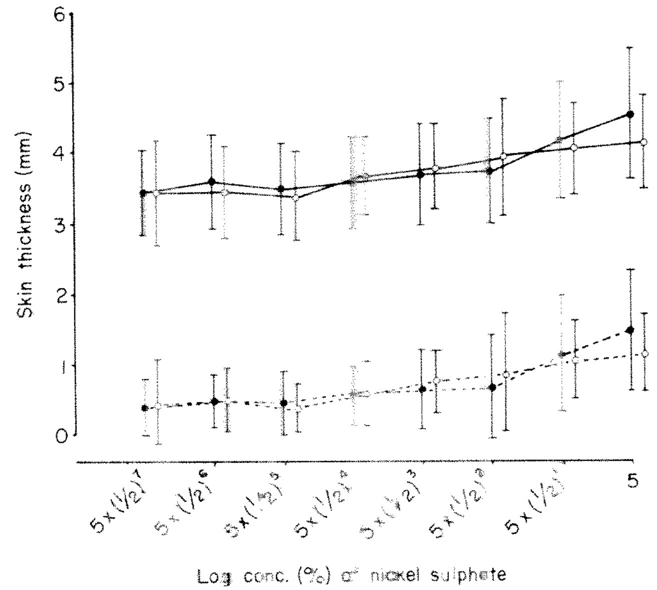

FIGURE 1. Dose–response curves according to the different concentrations of nickel sulphate by measurement of skin thickness using ultrasound. Absolute values of skin thickness were positively correlated with the log of nickel concentration 2 (●——●) and 4 (○——○) days after application. At 2 days skin thickness $= 3·93 + 0·21 \times$ (log[nickel concentration]), $r = 0·40$, $P < 0·001$. At 4 days, skin thickness $= 3·86 + 0·17 \times$ (log[nickel concentration]). Relative values (absolute value − normal skin value) were also positively correlated to the log of nickel concentration, 2 (●– – –●) and 4 (○– – –○) days after application. At 2 days increase in skin thickness $= 0·88 + 0·20 \times$ (log[nickel sulphate concentration]), $r = 0·46$, $P < 0·001$. At 4 days, increase in skin thickness $= 0·84 + 0·17 \times$ (log[nickel sulphate concentration]).

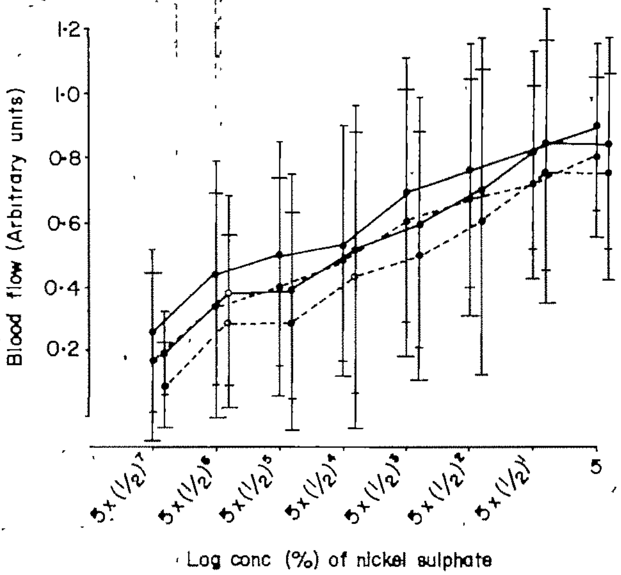

FIGURE 2. Dose–response curves according to the different concentrations of nickel sulphate as measured by laser-Doppler velocimetry. Absolute values were positively correlated with log of nickel concentration at 2 days: blood flow $= 0{\cdot}72 + 0{\cdot}13 \times (\log[\text{nickel concentration}])$, $r = 0{\cdot}53$, $P < 0{\cdot}001$, and at 4 days: blood flow $= 0{\cdot}58 + 0{\cdot}14 \times (\log[\text{nickel concentration}])$, $r = 0{\cdot}52$, $P < 0{\cdot}001$. For key see Figure 1.

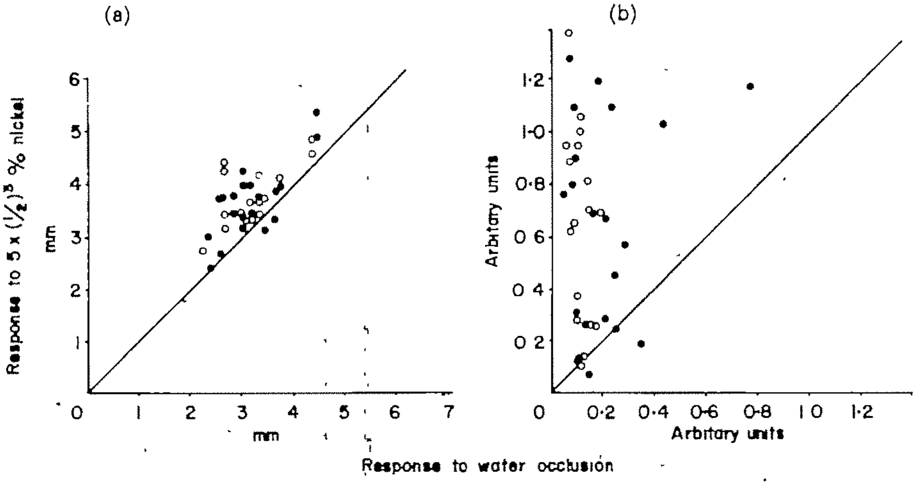

FIGURE 3. Scatter diagrams of the response measured by (a) ultrasound or (b) laser-Doppler method to $5 \times (\frac{1}{2})^5$% nickel against the corresponding response to water under occlusion. Nearly all points are above the diagonal line of equality, indicating a positive difference due to the nickel sulphate. ●, 2 days; ○, 4 days.

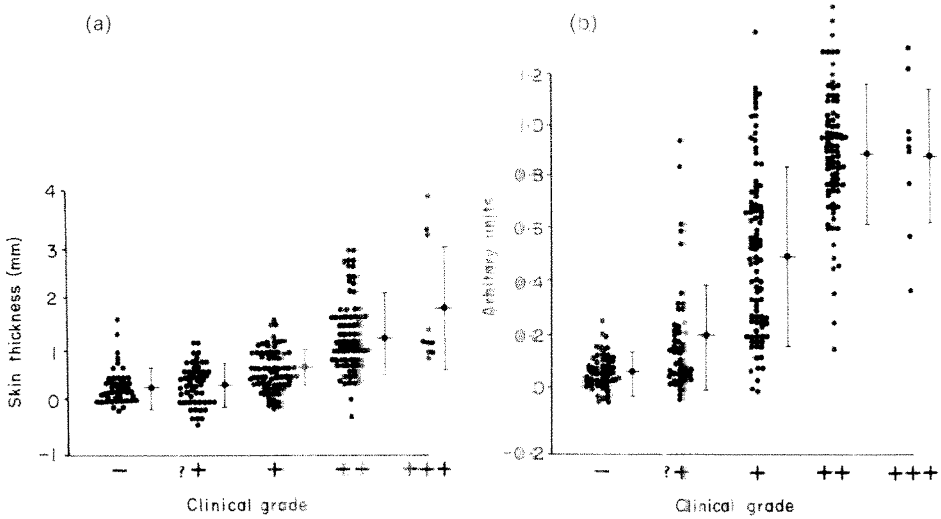

FIGURE 4. Scatter diagram showing (a) relative skin thickness and (b) relative blood flow measurements after nickel sulphate challenge in groups graded on their clinical reaction. Individual values (●) and mean ± SE (⧲) are shown. A high degree of overlap can be seen between the clinically graded groups.

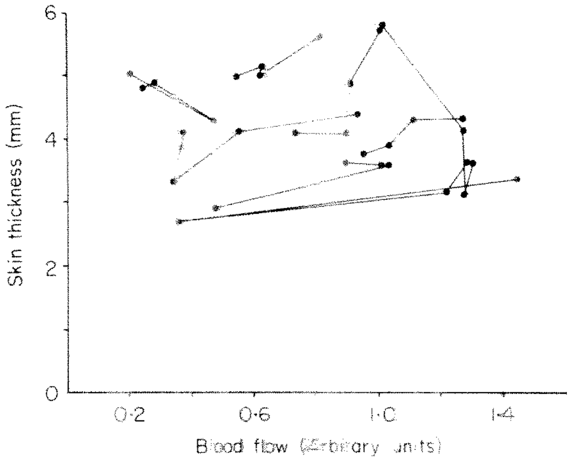

FIGURE 5. Correlation between ultrasound and laser-Doppler measurements in eight potassium dichromate-sensitive patients ($0{\cdot}5\% - 0{\cdot}5 \times (\frac{1}{2})^3\%$ only).

to nickel sulphate using ultrasound is shown in Figure 4(a) and the laser-Doppler flowmeter measurements Figure 4(b). The figure shows a high degree of overlap between the clinical grading groups. Similar scatter diagrams were observed with potassium dichromate in the potassium dichromate-sensitive patients.

DISCUSSION

This study shows that there is a relationship between the dose of antigen and the skin response in patch-test reactions. This differs from the concept of threshold sensitivity[16,17] or 'end-point', mainly because previous authors have only used a clinical evaluation. As shown in the scatter diagrams (Fig. 4), when the patch-test reactions are divided by the clinical grade there is a wide scatter of the objectively measured values within each group and extensive overlap between grades. This shows that subjective clinical grading is an inaccurate method when applied to the antigen dose–response of patch-test reactions.

Friedmann *et al.*[18] suggested that there was a log–dose response relationship in DNCB sensitized subjects and showed relatively linear log–dose response curves between different subgroups (sensitized by different DNCB doses). However, allergic patients in clinical practice are much more heterogeneous, as in our study, and there is a wide range of response patterns. The number of readings per patient, the large number of patients used, and their different ICD response types, all contribute to this. Another reason for the difference may be that our study covers a wider range of concentrations than was the case in Friedmann's study.

The study design can be criticised on the grounds that repeated measurements on each individual patient creates a within-patient and between-patient variance not accounted for in our simple linear model. We have re-analysed the data and shown that this criticism does not hold for the nickel sulphate sensitive patients' ultrasound and laser-Doppler measurements (see Statistical Appendix).

Non-invasive techniques such as pulsed A-scan ultrasound and laser-Doppler flowmetry may detect patients who have contact allergy but have negative or doubtful reactions on routine patch testing, because both instruments can differentiate between control and patch test responses over certain dilution ranges (Fig. 3). Objective measurements can also be useful in experimental animal or human sensitization studies,[18,19] because these methods are more sensitive than clinical scoring and give quantitative data when the response is clinically undetectable.

The correlation between the pulsed A-scan ultrasound and the laser-Doppler measurements in the same patients is weak, as shown in Figure 5. The two measurements are basically quite different as shown in the scatter diagrams (Fig. 3). With the laser-Doppler flowmeter the value for normal skin is quite small compared to the change after inflammation, in comparison with the change in ultrasound readings. For this reason it is desirable to calculate the relative value (absolute value − value of normal skin) when using ultrasound measurements. Even with objective measurements, evaluation of patch-test reactions is difficult. In addition to individual variations there is inconsistency in the response to different concentrations of antigens, with at times greater reaction to weaker concentrations and vice versa (patch to patch variation), contributing to wide standard deviations. There is also a recent report[20] that 40% of patch-test positive reactions are not reproducible on the opposite side of the back in the same patients. Clearly more standardized patch-test methods (such as True-Test)[21] should be developed alongside more sensitive and objective measurement techniques for the evaluation of patch-test reactions.

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STATISTICAL APPENDIX

The data for nickel-sensitive patients has been re-analysed using a multiple linear regression model (Statgraphics Software Package) to test whether between-subject and within-subject variation was equal.[1]

$$\text{Response} = \mu + \alpha + \pi + \beta + \alpha\beta + \beta\pi + \Sigma;$$

where: μ = mean;
α = variance due to $\log[\mathrm{Ni}^{2+}]$;
π = variance due to patient within $\log[\mathrm{Ni}^{2+}]$;
β = variance due to day of assessment;
$\alpha\beta$ = variance due to $\log[\mathrm{Ni}^{2+}]$*day interaction;
$\beta\pi$ = variance due to patient within day of assessment;
Σ = error variance.

According to Winer,[1] between subject variance:

$$\text{between subject variance} = \Sigma + (\pi \times \text{repeated measures});$$
$$\text{within subject variance} = \Sigma + \beta\pi.$$

For skin thickness the values for the coefficients generated were:

$\alpha = 26{\cdot}17$ $\alpha\beta = 0{\cdot}246$
$\pi = 0{\cdot}133$ $\beta\pi = 2{\cdot}36$
$\beta = 0{\cdot}001$ $\Sigma = 99{\cdot}50$

$$\therefore \text{between subject variance} = 100{\cdot}56$$
$$\text{within subject variance} = 101{\cdot}86.$$

For blood flow:

$\alpha = 14{\cdot}128$ $\alpha\beta = 0{\cdot}014$
$\pi = 0{\cdot}014$ $\beta\pi = 3{\cdot}379$
$\beta = 0{\cdot}333$ $\Sigma = 34{\cdot}79$

$$\therefore \text{between subject variance} = 34{\cdot}9$$
$$\text{within subject variance} = 38{\cdot}2.$$

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Histamine release from rodent and human mast cells induced by protoporphyrin and ultraviolet light: studies of the mechanism of mast-cell activation in erythropoietic protoporphyria

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SUMMARY

We report that protoporphyrin (PP) and ultraviolet light (UVA) induces histamine release from rat peritoneal mast cells, mouse bone marrow mast cells and human cutaneous mast cells in a dose- and temperature-dependent manner. The mast-cell activation was associated with loss of membrane integrity and inhibited by the hydrogen peroxide scavenger, catalase. Histamine release was independent of extracellular calcium in the rodent mast cells, but was markedly reduced in the absence of calcium in human cells. These findings indicate that PP and UVA induce mast-cell-mediator release by a process that may involve hydrogen peroxide formation. There appear to be differences in response to PP and UVA between rodent and human mast cells.

Erythropoietic protoporphyria (EPP) is an inherited disease associated with decreased activity of the enzyme ferrochelatase,[1,2] which results in elevated levels of protoporphyrin (PP) in the red blood cell, plasma, and faeces.[3] On exposure to sunlight, patients with EPP develop a burning and itching sensation followed within hours by oedema,[4] and these symptoms and signs of phototoxicity are consistent with those induced by mast-cell mediators.[5] Degranulation of the mast cells of the skin was thought to play an early role in the cutaneous manifestations of EPP. The findings on histology of mast-cell degranulation in early skin lesions of EPP appeared to confirm this,[6] and in animal models, functionally intact mast cells were found to be necessary for the full manifestation of porphyrin-induced photosensitivity.[7,8] Although other mediators of inflammation, in particular the complement system, are almost certainly involved in the acute

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response to sunlight, mast-cell products have been specifically implicated in the pathogenesis of the lesions of EPP.[9,10]

In one recent investigation of the role of mast-cell mediators in EPP, rodent mast cells preloaded with ^{3}H-serotonin and ^{14}C-arachidonic acid were induced to release these exogenous mediators by the addition of protoporphyrin (PP) and exposure to UVA light.[9] However, the release of endogenous mast-cell mediators was not studied, and rodent data may not necessarily be applicable to human mast-cell pathophysiology.[11] Therefore, in this study, the effect of PP and UVA on human skin mast cells was investigated and the responsiveness of this cell type compared to that of rodent cells. Histamine release was chosen as a measure of mast-cell activation because it is present in both rodent and human mast cells, and because histamine release has been observed in an *in vivo* animal model of porphyria and correlates well with phototoxic symptoms such as oedema.[10]

METHODS

Materials

The following chemicals were purchased from Sigma Chemical Co., St Louis, MO, U.S.A.: Bovine serum albumin (BSA), concanavalin A, collagenase (Type 1), hyaluronidase (Type 1), toluidine blue, trypan blue 0·4% in 0·9% NaCl, theophylline, compound 48/80, antimycin A, 2-deoxyglucose, mannitol, bovine superoxide dismutase (3100 U/mg), bovine liver catalase (11 000 U/mg), and sodium azide. L-glutamine, penicillin, streptomycin, non-essential amino acids, and heat-inactivated fetal calf serum were obtained from Gibco Laboratories, Grand Island, NY, U.S.A. Cromolyn was the gift of Dr Roger Menendez, Fisons Pharmaceuticals, Bedford, MA, U.S.A. Flow Laboratories, McLean, VA, U.S.A. was the source for the RPMI 1640 medium, and BioRad of Richmond, CA, U.S.A. for the 2-mercaptoethanol. All mice used were from MTS Laboratories, San Diego, CA, U.S.A. Sprague–Dawley rats came from Charles River Breeding Lab., Inc., Wilmington, DE, U.S.A.

Protoporphyrin IX (PP) was obtained from Porphyrin Products, Logan, UT, U.S.A. Protoporphyrin (1 mg) was dissolved in 1 ml of 11·6 N HCl and diluted 1/10 in distilled water to yield a stock solution of 100 μg/ml. The stock solution was stored in the dark at 4°C as 100 μl aliquots, which were further diluted in 19·9 ml of HEPES-buffered Tyrode's solution (TYR), pH 7·4, containing : NaCl (137 mM), D-glucose (5 mM), HEPES (10 mM), KCl (2·7 mM), NaH_2PO_4 (0·4 mM).

Isolation, growth, and maintenance of mast cells

Histamine release was studied using three cell systems: interleukin-3-dependent mouse mast cells differentiated *in vitro* from bone marrow precursors (BMMC), rat peritoneal mast cells (RPMC), and mast cells isolated from human infant foreskin (HSMC).

To obtain BMMC, bone marrow cells were harvested from the femurs of 6–8 week old BALB/c mice. They were cultured in RPMI 1640 medium (Dutch modification) with the addition of L-glutamine (4 mM), 2-mercaptoethanol (50 μM), penicillin (50 U/ml), streptomycin (50 mg/ml), non-essential amino acids (100 μM each acid), and supplemented with 10% heat-inactivated fetal calf serum. The supernatant from a cell culture line of WEHI-3B cells (a line of mouse myelomonocytic cells that constitutively produce interleukin-3) was also added at a final concentration of 25%. The cells were maintained in liquid culture at a temperature of 37°C and in a humidified atmosphere of 5% CO_2 and 95% air. They were routinely passaged at weekly intervals by dividing the culture and adding fresh medium supplemented as described above. BMMC were used after 3–6 weeks of culture.

RPMC were obtained by peritoneal lavage of 200–400 g male Sprague–Dawley rats with 20 ml of TYR containing heparin (10 U/ml) and BSA (2 ml of 38% BSA). After centrifugation, the cells were purified on a 38% BSA gradient, yielding a final population of > 99% viable cells as assessed by trypan blue staining and of > 95% pure mast cells as assessed by toluidine blue staining.[12]

A human skin mast-cell suspension was obtained from human infant foreskin specimens using the method described by Benyon *et al.*[13] The skin was coarsely chopped, then washed in HEPES-buffered Hanks' balanced salt solution (HBSS), pH 7·4, containing: NaCl (137 mM), HEPES (20 mM), D-glucose (5 mM), KCl (2·7 mM), NaH_2PO_4 (0·4 mM), $MgCl_2$ (0·5 mM), $CaCl_2$ (1 mM), and fetal calf serum (1·5%). Chopped foreskin was incubated in HBSS containing collagenase (1·5 mg/ml) and hyaluronidase (0·5 mg/ml) at 37°C for 60 min. Dispersed cells were harvested by filtration through nylon gauze (150 μm pore size) and washed twice in HBSS by centrifugation (200 *g*, 5 min, 4°C). The undigested tissue was subjected to a second identical digestion and separation, then further dispersed with a syringe and filtered through cotton gauze. After washing by centrifugation both sets of cells were pooled and incubated at room temperature overnight in medium identical to that used for BMMC culture, except that no WEHI supernatant was added. Within 24 h of their enzymatic dispersion, HSMC were used as a mixed cell suspension, in which they represented approximately 5% of the total cell population. Viability of the cell preparation as assessed by trypan blue exclusion was > 95%.

Light source and irradiation of mast cells

Four General Electric F40BL tubes were used as the source of UVA light. The lamps emitted the majority of photons over a wavelength range of 320–450 nm, with peak emission at 350 nm. The output of the lamps at wavelength range 390–410 nm was 50 $\mu W/cm^2$, measured at a distance of 15 cm, with an IL Research Radiometer, a SEE400 sensor, a NB405 filter, and a W1968 diffuser (International Light, Newburyport, MA, U.S.A.). A 3-mm windowglass filter was used during irradiation to eliminate any possible photons of wavelengths below 320 nm. The doses of irradiation used were 60–320 J/m^2, as measured at 390–410 nm, the peak region of the absorption spectrum of PP. These doses are similar to those obtained during outdoor activity[14,15] and therefore are clinically relevant.

The RPMC and BMMC to be used experimentally were washed three times in TYR, and resuspended to concentrations of $2{\cdot}5 \times 10^5$ cells/ml and $2{\cdot}5 \times 10^6$ cells/ml, respectively. HSMC in the cell preparation dispersed from human skin were washed once and used without further purification, at a concentration of $7{\cdot}5 \times 10^5$ cells/ml. Aliquots (200 μl) of each type of mast cell were then added to polystyrene tubes containing either PP in TYR (200 μl) or TYR alone (200 μl). Final PP concentrations studied were in the range of 30–250 ng/ml. These concentrations of PP correspond to those reported in the skin of patients with EPP.[16] In some experiments, which are specified in the legends, calcium and magnesium were present in the reaction mixture at a concentration of 1 mM each. Otherwise, all experiments were performed in calcium- and magnesium-free buffer. In experiments with metabolic inhibitors, glucose-free buffer was used. Immediately after the cells were added to the polystyrene tubes, they were placed in a dark water-bath for 10 min at the desired temperature. Cells were irradiated in the same polystyrene tubes and at the same temperature at a distance of 15 cm from the light source. The temperature was continuously monitored. Controls consisted of cells irradiated in buffer alone, and unirradiated cells in buffer with or without PP. Duplicate samples were prepared for each

TABLE 1. Trypan blue staining of rat peritoneal mast cells

Staining grade	Staining of nucleus/cytoplasm	Cell morphology
0	−/−	Normal
1	+*/−	Normal
2	+/−	Cells swollen with vacuoles
3	+/−	Cell membrane disrupted

* Nuclear staining was pale.

experiment. Immediately after irradiation, both irradiated and control cells were inhibited from further histamine release by the addition of 2·6 ml of ice-cold TYR. Each tube was then centrifuged at 300 ***g*** for 5 min. Samples were separated into supernatants and pellets, and the pellets were resuspended in TYR to a volume of 3 ml and sonicated. The histamine content of all samples was then measured by a manual fluorimetric assay, following formation of a fluorescent adduct with *o*-phthaldialdehyde.[17] The release of histamine into the supernatant was calculated as a percentage of that initially present in the cells. In each experiment results were expressed as per cent net release, which equals per cent total release minus per cent release in the presence of buffer alone. While cromolyn, theophylline, mannitol, and sodium azide did not interfere with the manual fluorimetric histamine assay at the concentrations used in these experiments, catalase did. Therefore, in experiments involving catalase, a manual histamine extraction[18] was performed on all samples prior to using the fluorimetric assay.

In some experiments the integrity of the mast-cell membrane was assessed. Cell suspension (20 μl) was added to 20 μl of 0·4% trypan blue, and dye uptake by 100 cells was assessed on a Neubauer haemocytometer. Cellular staining was graded as follows (Table 1): 0, no uptake of trypan blue; 1, pale nuclear staining with otherwise normal cellular morphology; 2, diffuse cellular staining with cell swelling and vacuole formation; 3, cellular staining with visible disruption of the cell membrane or a deeply stained free cell nucleus without associated cytoplasm. Grades 0 and 1 were considered to represent intact cells.[19]

Statistical evaluation

All data are expressed as means ± standard error of the mean (SEM) for a given number (n) of experiments. The significance of differences between experimental groups was assessed using Student's t-test for unpaired samples, with values of $P < 0{\cdot}05$ considered to represent a significant difference.

RESULTS

Effect of PP and irradiation on the release of histamine

In the presence of PP, exposure to UVA irradiation resulted in the release of histamine from all three cell types examined (Fig. 1). All three cell types showed greater histamine release as the concentration of PP was increased. Additionally, both mouse BMMC and RPMC showed a clear relationship between the dose of irradiation and the release of histamine, with maximum per cent net histamine release occurring at the highest UVA dose. HSMC were not examined at varying UVA intensities due to limited availability of cells. BMMC and RPMC, in the presence of PP (250 ng/ml) and exposed to 320 J/m^2 of 390–410 nm irradiation, produced a maximal net histamine release of 43·6 ± 5·1% and 52·1 ± 2·0%, respectively (Fig. 1 a, b). In the absence of PP and UVA, the background spontaneous histamine release was < 5% for both RPMC and BMMC. Irradiation alone resulted in < 5% net release for RPMC and < 12% for BMMC. Incubation with PP in the dark produced < 5% net release for both cell types. HSMC gave a maximum net histamine release of 30·5 ± 2·9% (Fig. 1c) and a spontaneous release that was usually < 15%.

Effect of temperature

When BMMC, RPMC, and HSMC were incubated with PP (250 ng/ml) for 10 min and then irradiated with UVA (320 J/m^2) at varying temperatures, histamine release by all cell types was suppressed at 0°C, lowered at 30°C, and slightly elevated at 45°C relative to the release observed at 37°C (Fig. 2). The temperature dependency was not altered by increasing the amount of PP added (data not shown).

Effect of scavengers of reactive oxygen radicals

The role of reactive oxygen radicals was studied by exposing RPMC and HSMC to PP (250 ng/ml) and irradiation (320 J/m^2) in the presence or absence of mannitol, sodium azide, or catalase. Histamine release was 54·6 ± 1·5% and 38·2 ± 2·7% from RPMC and HSMC, respectively, in the absence of scavengers (Table 2). Mannitol (10 mM), a hydroxyl radical scavenger, and sodium azide (0·1 mM), a scavenger of singlet oxygen, produced no suppression of histamine release from either cell type. Catalase (3438 U/ml), a scavenger of hydrogen peroxide, produced 61·7 ± 2·6% suppression of the release of histamine from RPMC and 79·8 ± 1·7% from HSMC ($P < 0·001$). This inhibition was dose-dependent and was consistent with the previously observed *in vitro* inhibition of PP- and UVA- induced serotonin release from mast cells.[9]

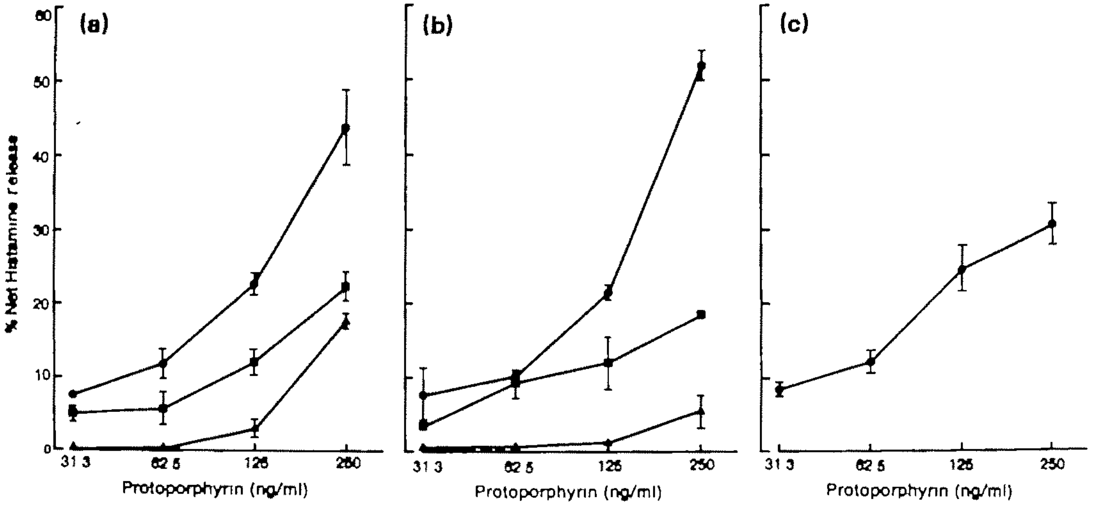

FIGURE 1. The effect of varying concentrations of PP on histamine release from (a) BMMC, (b) RPMC and (c) HSMC at different doses of UVA light. ●——●, 320 J/m^2; ■——■, 220 J/m^2; ▲——▲, 60 J/m^2. Results are given as means ± SEM, $n = 4$ (BMMC, RPMC), $n = 12$ (HSMC).

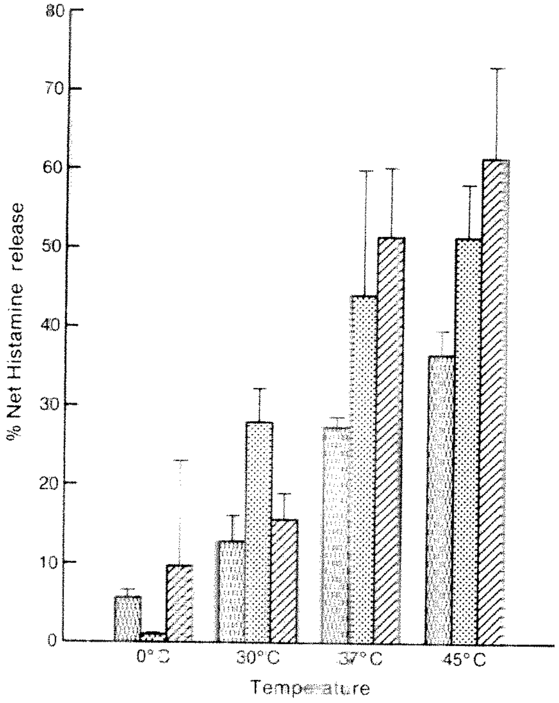

FIGURE 2. The effect of temperature on the release of histamine from HSMC, BMMC, and RPMC by PP (250 ng/ml) and UVA (320 J/m².) Values are means ± SEM for at least four experiments. ▩, HSMC; ▦, BMMC; ▨, RPMC.

TABLE 2. Effect of scavengers of reactive oxygen radicals on histamine release induced by protoporphyrin (250 ng/ml) and irradiation (320 J/m²) from RPMC and HSMC

Scavengers	Net % release ± SEM*		Per cent suppression
	Without	With	
RPMC			
Mannitol (10 mM)	62·4 ± 1·2	57·6 ± 3·9	7·7
Sodium azide (0·1 mM)	53·4 ± 1·3	61·0 ± 0·9	− 12·6
Catalase (3438 U/ml)	50·6 ± 4·3	13·7 ± 4·8	72·9
HSMC			
Mannitol (10 mM)	30·2 ± 0·9	30·3 ± 1·6	0·6
Sodium azide (0·1 mM)	37·1 ± 3·4	36·9 ± 1·6	− 1·3
Catalase (3438 U/ml)	43·9 ± 0·6	16·8 ± 0·8	61·7

The net release induced by the scavengers alone was: RPMC; mannitol, 6·1 ± 0·1; sodium azide, 6·1 ± 1·0; and catalase, 6·9 ± 11 (mean ± SEM). HSMC; mannitol, 10·5 ± 1·3; sodium azide, 14·4 ± 0·6; and catalase, 14·3 ± 0·2.

* Separate experiments were performed with each scavenger. The results represent the mean of at least two experiments, each performed in duplicate.

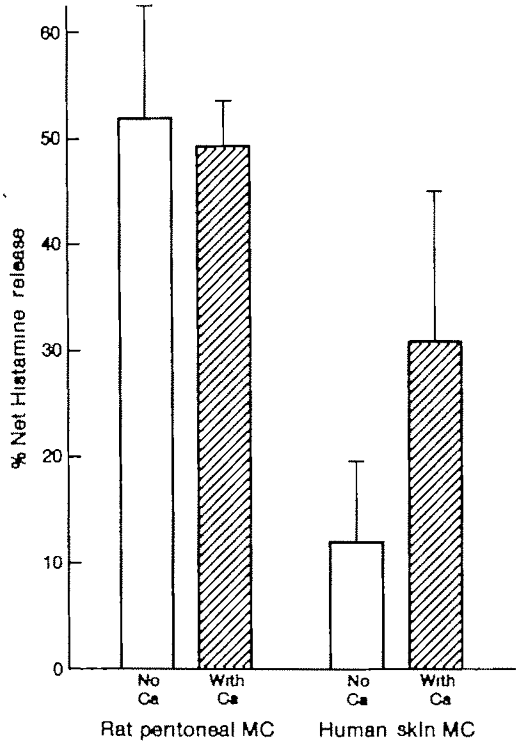

FIGURE 3. Histamine release induced from mast cells by PP (250 ng/ml) and UVA (320 J/m^2) in the absence (□) or presence (■) of added calcium and magnesium ions. Values are means ± SEM for 4–6 experiments. Release from human skin MC is significantly higher in the presence of calcium and magnesium than in their absence ($P < 0.05$).

Effect of divalent cations

RPMC incubated with 250 ng/ml PP and exposed to UVA produced 51·9 ± 5·2% net histamine release in buffer without calcium or magnesium and 49·3 ± 2·4% when these ions were added at concentrations of 1 mM each (Fig. 3). In contrast, under identical conditions, HSMC released 12·1 ± 3·4% of their histamine without calcium and magnesium and 30·8 ± 5·7% when these ions were present ($P < 0{\cdot}05$).

Kinetics

As shown in Figure 4, histamine release from RPMC was dependent upon the incubation time of the cells with PP prior to irradiation. To examine whether histamine release was the result of incorporation of PP into cells, RPMC were incubated with PP (250 ng/ml) for 10 min at 37°C, washed three times with ice-cold TYR and irradiated (320 J/m^2). Duplicate samples were irradiated without washing. Similar net release of histamine was obtained when cells were irradiated without washing (59·3 ± 1·1%) or following removal of PP from the medium by washing (62·8 ± 2·4%). These results suggest that a time-dependent process, perhaps reflecting incorporation of the PP molecules into the mast cell, is important in photoactivation.

Membrane integrity of PP and UVA-treated mast cells

The capacity of PP and UVA to induce alterations in mast-cell membrane integrity and to

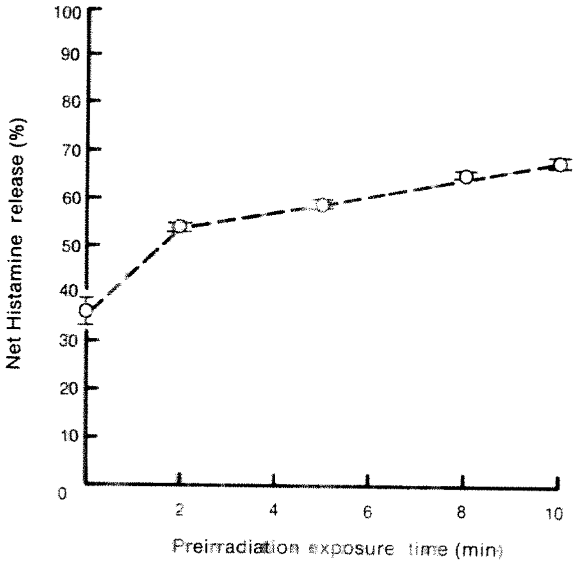

FIGURE 4. Histamine release as a function of the incubation time of RPMC with PP. Cells were incubated with PP 250 ng/ml for increasing times prior to irradiation with 320 J/m^2. Values are means ± SEM for four experiments. Each incremental increase in pre-incubation time resulted in a significantly higher release of histamine ($P < 0·05$ or better).

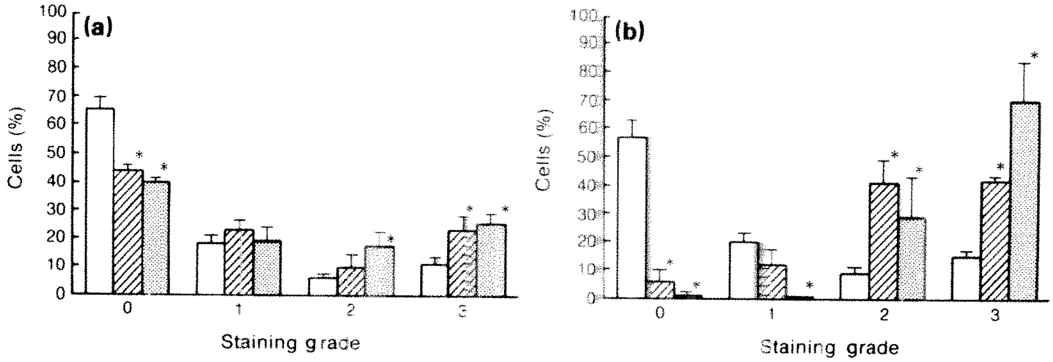

FIGURE 5. Trypan blue staining of RPMC incubated either with buffer alone □, PP at 25 ng/ml ▨, or PP at 250 ng/ml ▩. (a) Staining of unirradiated cells; (b) Staining of cells irradiated with 320 J/m^2. Values are means ± SEM for 2–9 experiments. Asterisks indicate a significant difference ($P < 0·05$) between cells exposed to PP and those in buffer alone for each staining grade in either (a) or (b). For an explanation of staining grades, see Table 1.

produce histamine release were compared. Membrane integrity was assessed by the ability of the cells to exclude trypan blue (Table 1). As shown in Fig. 5a, the majority of RPMC incubated in the absence of PP and UVA were either unstained by trypan blue (grade 0; 65·9 ± 3·8%) or showed pale nuclear staining with normal cellular morphology (grade 1; 17·7 ± 2·7%). Exposure of the cells to PP (25 ng/ml) with irradiation (Fig. 5b) significantly altered this pattern. Under these conditions, only 18·0 ± 8·9% of the cells maintained cellular integrity (5·8 ± 3·8%, grade 0 and 12·3 ± 3%, grade 1). The majority of the cells showed evidence of cell damage with either diffuse cellular staining (grade 2, 40·8 ± 7·9%) or visible disruption of the cell membrane (grade 3, 41·8 ± 1·0%), despite the fact that the net histamine release was only 12·6 ± 3·3%. With a higher concentration of PP (250 ng/ml) and irradiation, there was evidence of increased cell damage (29·2 ± 13·7%, grade 2 and 69·4 ± 13·7%, grade 3), with few, if any, intact cells. Net histamine release induced by UVA and this higher dose of PP was 67·8 ± 1·6%.

There was a significant alteration in the integrity of RPMC membranes induced by PP in the absence of UVA irradiation. Fewer cells treated with PP at 250 ng/ml displayed grades 0 or 1 (39·8 ± 0·6%, grade 0 and 8·5 ± 4·7, grade 1) compared with untreated cells (65·9 ± 3·8%, grade 0 and 17·7 ± 2·7%, grade 1), while a significantly higher proportion of the PP-treated cells exhibited staining grades 2 and 3 (17·0 ± 5·1% and 2·4 ± 4·4%). Similar results were obtained when 25 ng/ml of PP were used. These decreases in cell membrane integrity in RPMC treated with PP without irradiation were observed in the absence of any histamine release above that of controls. UVA irradiation in the absence of PP had no effect on trypan blue staining of the cells ($n=2$).

Effect of metabolic inhibitors

The combination of antimycin A (1 μM) and 2-deoxyglucose (10 mM), inhibitors of oxidative phosphorylation and glycolysis, respectively, had no effect on histamine release from either RPMC or HSMC when these cells were exposed to PP (250 ng/ml) and UVA (320 J/m^2). Histamine release was 76·0 ± 5·0% (RPMC), 56·7 ± 2·6% (HSMC) and 71·3 ± 1·8% (RPMC), 42·9 ± 2·8% (HSMC) in the presence and in the absence of the inhibitors. In control experiments, 20 min of incubation with the combined inhibitors completely inhibited release induced from RPMC by concanavalin A (22·3 ± 1·5 vs. −5·5 ± 0·5%) and by compound 48/80 from HSMC (11·5 ± 0·5% vs. 0·0 ± 1·0%). The DMSO vehicle used to dissolve antimycin A (final concentration 0·25%) had no effect on either basal or stimulated histamine release (data not shown).

Effect of cromolyn and theophylline

Cromolyn is an inhibitor of both metabolic and cytotoxic histamine release from mast cells.[20] Cromolyn (0·1 mM) failed to inhibit histamine release from RPMC induced by PP (250 ng/ml) and UVA (320 J/m^2). Histamine release was 56·0 ± 6·7% in the absence of cromolyn and 60·0 ± 6·7% when cromolyn was added to the cells 10 min prior to irradiation ($n=4$). Similarly, the drug did not inhibit histamine release when it was added immediately prior to irradiation. The same concentration of cromolyn produced significant inhibition of histamine release induced by compound 48/80 (1 μg/ml) as the release of histamine was 29·0 ± 3·2% and 38·1 ± 2·5% in the presence and absence of cromolyn, respectively ($P < 0{\cdot}05$).

Theophylline is a known inhibitor of both cytotoxic-and IgE-mediated histamine release from mast cells.[20,21] The photoactivation of RPMC by PP (250 ng/ml) and UVA (320 J/m^2) was not inhibited by theophylline (10 mM). In the presence of this drug, histamine release was 76·1% ± 0·72%, compared with 68·4 ± 1·5% in its absence ($n=6$). This concentration of theophylline was adequate to completely inhibit the histamine release from RPMC induced by concanavalin A (10 μg/ml), in which case histamine release was 0·4 ± 1·4% and 11·4 ± 2·3% in the presence and absence of theophylline, respectively ($P < 0{\cdot}05$).

DISCUSSION

This study investigated whether the clinical manifestations of EPP could be partly explained by the effect of porphyrin and light on the human mast cell. The properties of HSMC in this system were compared with those of BMMC and RPMC which had previously been shown to release exogenous mediators following PP + UVA treatment.[9] Exposure to PP and irradiation resulted in a release of endogenous histamine from BMMC and RPMC which was dependent upon the concentration of PP, as well as the irradiation dose. Similarly, HSMC irradiated at 320 J/m^2 showed increasing histamine release with rising concentrations of PP, demonstrating that mast cells from all three species release histamine upon exposure to UVA irradiation and PP.

Net histamine release by PP and UVA from all three cell types was low at 0°C and was slightly increased at 45°C compared to that observed at 37°C. The lack of inhibition at 45°C suggested that a cytotoxic rather than an enzymatic process was involved.

The role of reactive oxygen radicals, which was previously studied in PT18 cells preloaded with [^{3}H]-serotonin,[9] was studied in RPMC and HSMC using scavengers specific for the various oxygen species. Only catalase had a protective effect, implying a role for hydrogen peroxide in the PP- and UVA-induced release of histamine from both cell types. These findings agree with studies which have shown PP- and UVA-induced damage of the erythrocyte membrane, accompanied by formation of hydrogen peroxide.[22,23] Photoinduced histamine release may be the result of similar membrane damage of mast cells produced by the liberation of hydrogen peroxide, but it remains to be proven whether hydrogen peroxide is a direct releasing stimulus.[24]

The effect of calcium on histamine release induced by PP and UVA was investigated using RPMC and HSMC in the presence or absence of calcium and magnesium. While magnesium can substitute for calcium as a second messenger for histamine release when there is no calcium available, calcium is the normal second messenger for secretion following immunologic stimulation. RPMC exhibited similar net histamine release with or without the addition of calcium to the buffer. In contrast, histamine released by HSMC in the absence of calcium was $< 50\%$ ($12{\cdot}1 \pm 3{\cdot}4\%$) of that observed in the presence of calcium ($30{\cdot}8 \pm 5{\cdot}7\%$). These data suggest that a different mechanism may operate in the phototoxic release of histamine from HSMC and RPMC.

By incubating RPMC with PP for increasing times prior to irradiation, we demonstrated that histamine release was dependent on the length of this exposure. This suggests that the ability of PP to produce photoactivation of mast cells may relate to cell-membrane solubility.[9, 25] RPMC incubated with PP for 10 min and then washed prior to irradiation were found to release the same amount of histamine as unwashed cells, which again suggests diffusion of lipid-soluble PP molecules into the mast-cell membrane prior to photoactivation by UVA.

Direct staining of RPMC after exposure to PP and UVA indicated that the photoactivation process is associated with a loss of membrane integrity. This finding agrees with previous studies[9, 26] which showed photolysis of rodent mast cells under similar conditions. Trypan blue staining may be a more sensitive indicator of mast-cell photoactivation than histamine release, as demonstrated by the staining changes seen with 25 ng/ml of PP and UVA, in the absence of marked histamine release. The mechanism of cytotoxicity is, however, dissimilar from that produced by detergents. Theophylline and cromolyn, at concentrations sufficient to inhibit metabolic activation of mast cells, failed to protect both human and rodent mast cells from PP-induced photolysis, even though the concentrations of these drugs used have been reported to be sufficient to inhibit surfactant-induced mast-cell lysis.

The use of a mixed cell suspension as the HSMC preparation should be addressed. While mast cells are the only cells in the experimental mixture which release histamine, such release does not necessarily indicate a primary effect on the mast cells. However, this study has demonstrated histamine release in HSMC exposed to UVA and PP *in vitro*. Although previous studies have demonstrated exogenous mediator release from rodent mast cells in conditions simulating EPP, the ability of human skin mast cells to release endogenous mediators in response to clinically relevant doses of PP and UVA had not previously been addressed. Similar endogenous histamine release may occur *in vivo* in the cutaneous mast cells of patients with EPP.

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6-Methylangelicins: new monofunctional photochemotherapeutic agents for psoriasis

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SUMMARY

The monofunctional furocoumarins, the 6-methylangelicins, were tested for their antiproliferative activity with various animal models and for genotoxicity in micro-organisms and in mammalian cells. The most active compound was 6,4,4′-trimethylangelicin, which showed a high antiproliferative effect and reduced genotoxicity in comparison with 8-methoxypsoralen (8-MOP). Some of these compounds were also tested clinically by topical application on 17 patients with psoriasis. They appeared to be more active than 8-MOP in clearing psoriasis without inducing skin phototoxicity. The methylangelicins also caused skin pigmentation.

The photochemotherapy of skin diseases is carried out by administering psoralen (a linear isomer of furocoumarins) plus UVA (at 320–400 nm) light (PUVA).[1] At present the drug most frequently used for this treatment is 8-methoxypsoralen (8-MOP)[1] although 5-methoxypsoralen (5-MOP)[2] and 4,8,5′-trimethylpsoralen (TMP)[3] are also employed.

The effectiveness of this therapy is mainly ascribed to specific photodamage to DNA in the epidermal cells, inhibiting DNA replication and therefore cell division.[1,4] In particular, the psoralen molecule, intercalated between two base pairs of DNA, photoconjugates with one chain forming monoadducts, or with both complementary chains forming inter-strand cross-linkages (diadducts).[5]

PUVA is considered to be a most effective therapeutic treatment for severe psoriasis and the early stages of mycosis fungoides, but there are some side-effects such as skin phototoxicity,

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pruritus, nausea and impairment of cell-mediated immunity.[1] The increased incidence of skin cancer in patients subjected to long-term PUVA treatment has also been reported.[6]

In order to reduce the risks of PUVA treatment, we prepared and studied various methylangelicins, monofunctional furocoumarins which do not photoinduce inter-strand cross-linkages in DNA but which photobind with macromolecules only as monoadducts.

Three series of methylangelicins were prepared: the first, 5-methyl, 4,5′-dimethylangelicin, showed less therapeutic activity than 8-MOP;[7] the second, the 4′-methylangelicins, showed good antiproliferative activity and a greater therapeutic effect than 8-MOP. However, these compounds showed some skin phototoxicity on guinea-pig and human skin.[8,9]

Another important problem related to the synthetic procedure of the above angelicin derivatives was their purity. The introduction of a methyl group in the 6-position seemed to be a good solution,[10] and a third series of compounds were therefore prepared: the 6-methylangelicins. Some of these efficiently photobind to DNA and show a good antiproliferative action.[10] Preliminary clinical results indicate that some 6-methylangelicins also show a marked therapeutic effect.[10,11]

This paper describes the effectiveness of the most active 6-methylangelicins, i.e. 6,4-dimethylangelicin (6,4-DMA), 6,4′-dimethylangelicin (6,4′-DMA), 6,4,5′-trimethylangelicin (6,4,5′-TMA) and 6,4,4′-trimethylangelicin (6,4,4′-TMA) (Fig. 1), in clearing lesions of psoriasis with topical application and UVA irradiation in 17 patients.

The photomutagenic activity of two of these compounds (6,4′-DMA and 6,4,4′-TMA) was also investigated and compared with 8-MOP on three different biological systems, i.e. *Escherichia coli* WP2 uvrA, Chinese hamster V79 cells, and *Aspergillus nidulans* conidia.

METHODS

UV sources

For evaluation of epidermal DNA synthesis inhibition in mouse, irradiation was carried out using Philips HPW 125 lamps. The quartz bulbs in these lamps were coated by nickel–cobalt Wood's filter and the emission spectrum was in the range 313–405 nm, with a maximum at 365 nm (over 90% of total emission).[12] Irradiance was determined by a chemical actinometer[13] and was 0·55 mW/cm^2. For photomutagenicity experiments, a 'Blacklite' blue fluorescent lamp (F 15 T8 BLB, 15 W) was used. The emission spectrum was in the range 320–400 nm. Irradiance, determined as described above, was 0·191 mW/cm^2. It was then routinely monitored using a UVX digital radiometer equipped with a UVX-36 sensor (Ultraviolet Products Inc., S. Gabriel, CA, U.S.A.).

The patients were irradiated with high intensity UVA emitted from a low-pressure mercury fluorescent lamp (mod. PUVA Waldmann Sylvania F15 T8). The emission spectrum was in the range 320–400 nm. Irradiance was 8 mW/cm^2 as determined by a calibrated photometer (International Light).

Toxicity

The lethal dose was determined on NCL mice (weight 20 ± 1 g), using 10 animals for every dose and with four dose levels. The mice were not fed for 4 h prior to oral administration of the drug which was suspended in 0·5% methylcellulose (0·2 ml/mouse). The calculations were carried out according to the method of Weil.[14]

Inhibition of epidermal DNA synthesis in mouse

The methods used were similar to those previously described.[15]

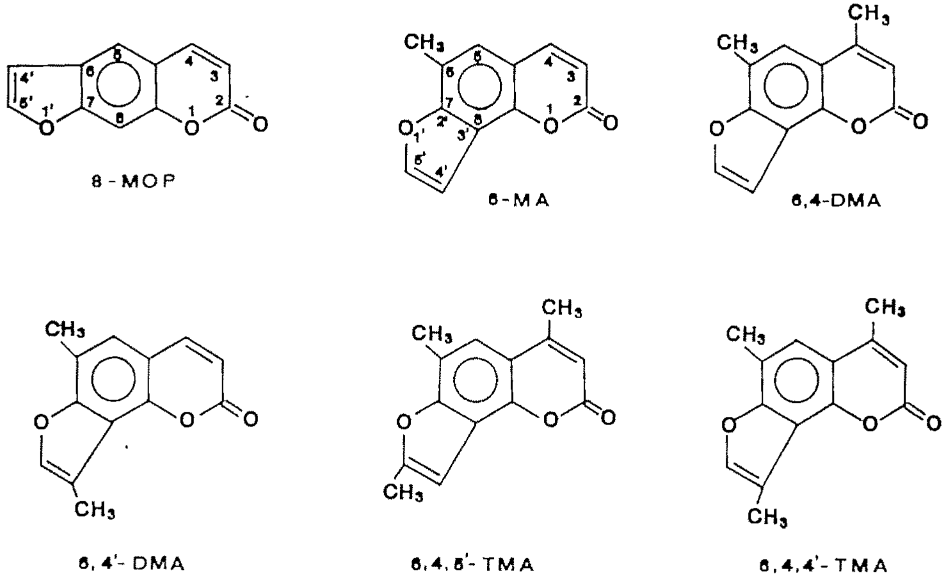

FIGURE 1. Molecular structure of 8-MOP, 6-MA, 6,4-DMA, 6,4'-DMA, 6,4,5'-TMA and 6,4,4'-TMA.

Topical application. Furocoumarins were applied to the depilated skin of the backs of female NCL mice using a 0·1% methanolic solution (50 μg/cm^2); each compound was tested on six mice. They were kept in a dark room for 45 min, after which their backs were irradiated at 365 nm (0·99 J/cm^2, delivered over 30 min). Immediately after irradiation, each mouse was injected intraperitoneally with 10 μCi (0·14 nM) of [^{3}H]-thymidine (specific activity 70 Ci/mM; Radiochemical Centre, Amersham, Bucks, U.K.) in Hanks' solution (0·2 ml) and killed 30 min later.

The skin specimens were removed and the epidermis isolated according to the method of Bowden *et al.*,[16] with slight modifications: the whole skin was kept for 1 min in distilled water at 55°C and chilled by immersion in ice-cold water. It was then placed on a cooled (−20°C) glass plate, dermis side down, and the epidermis removed by scraping with a razor blade. The epidermis was cut and suspended in 10 volumes of ice-cold sodium saline citrate (0·15 mol/l sodium chloride; 0·02 mol/l sodium citrate) and then homogenized in a Vortex mixer and a Potter–Elvehjn homogenizer, and cooled in ice. The mixture was composed of 2% sodium lauryl sulphate and 1 mol/l sodium chloride and left at room temperature for 2 h. The suspension was then extracted three times with a chloroform-butanol 4:1 mixture (v/v).[17]

The DNA was precipitated with a double volume of ethanol, washed with 80% ethanol and dissolved in 1 ml of water. The DNA content of the solution was determined (0·2 ml aliquots, by reaction with diphenylamine),[18] and radioactivity (0·5 ml samples, diluted to 1 ml of water, mixed with 10 ml of Instagel (Packard Instruments)) counted with a 300 CD Packard scintillation spectrometer.

Controls were skin specimens of the abdomen of the same animals not exposed to UVA light. Preliminary experiments showed that ventral and dorsal skin have similar levels of epidermal DNA synthesis. The specific activities observed in the control skin samples were in the range 0·9–1·2 × 10^4 d.p.m./mg of DNA.

Oral administration. The NCL mice (weight 20 ± 2 g) were not fed for 3 h prior to the oral administration of a single dose of 5 mg per mouse of the furocoumarin, suspended in 0·5% methylcellulose. Groups of at least six animals were used for each experiment. The mice were kept in a dark room for 2 h and the skin of the back was then irradiated with UVA (0·99 J/cm^2).

Subsequent steps for epidermal cell isolation, homogenization and DNA extraction were similar to those described above. The unexposed skin of the abdomen, protected from light by black paper, was used as a control, as described above.

Phototoxicity

Experiments on albino guinea-pig skin (weight 250–350 g). Measured volumes of 5 and 50 μg/cm^2 of 0·1% ethanol solution were placed by micropipette on a 4 cm^2 area of depilated skin. The solvent was rapidly evaporated by means of an air stream and the skin was irradiated for 1 h with a HPW 125 Philips lamp (total radiation dose 1·98 J/cm^2).[19] Visual gradings of the erythema responses were made 24 and 48 h after exposure.

Experiments on human skin of psoriatic patients. The experiments were carried out on areas of the skin not affected by psoriasis. The treated areas were observed 24 and 48 h after irradiation to evaluate the erythema response.

The scale adopted was that used by Fitzpatrick *et al.*[20] 0, no erythema; +, barely perceptible; + +, pink; + + +, red, with oedema; + + + +, marked erythema, oedema with vesiculation.

Pigmentation

The treated areas were observed 1 and 2 weeks after exposure to UVA and the pigmentation assessed as follows: 0, no tan; +, light tan; + +, medium tan; + + +, dark tan (after Fitzpatrick *et al.*[20]).

Photomutagenicity

The genotoxic action of 6,4′-DMA and 6,4,4′-TMA plus UVA was studied on three biological systems: the prokaryotic system, *Escherichia coli* WP2 uvrA and two eukaryotic systems, *Aspergillus nidulans* and V79 Chinese hamster cell. The genotoxic activity of 8-MOP plus UVA (320–400 nm) was used for a reference. In all the experiments, controls were carried out by keeping the micro-organisms in the dark in the presence of the drug and irradiating them in the absence of the drug.

Escherichia coli B, strain WP2 uvrA, grown in Davis Mingioli minimal salt glucose medium[21] plus 20 μg/ml tryptophan, was suspended at a density of 4 × 10^8 cells/ml in phosphate-buffered saline. The furocoumarins, dissolved in absolute ethanol, were added to a final concentration of 5 μg/ml of the drug and 0·025% of ethanol. Aliquots of 5 ml of the suspensions were poured onto 5-cm Petri dishes, to form a layer about 1 mm thick. They were kept in the dark at room temperature for 20 min and then irradiated with a 'Blacklite' blue fluorescent lamp. The irradiated cells were plated on SEM agar containing 1·5% of agar (fortified with 0·1 mg/ml of Difco nutrient broth) and revertant colonies were scored after 48 h of incubation at 37°C. Survivors were scored by plating appropriate dilutions of the irradiated cells in the same MMA medium supplemented with tryptophan.

Aspergillus nidulans haploid strain 35 was propagated in complete medium. The survival rates and mutagenicity were determined in minimal Czapek-Dox (CD) medium. After 90 min incubation in the dark at 37°C in distilled water containing 5 μg/ml of the furocoumarins and 0·025% of ethanol, used as a solvent for the drug, the conidia (8–10 × 10^7 ml^{-1}) were irradiated as described for *E. coli* cells. They were then plated on CD-medium containing 8-azaguanine (final concentration 1·5 mmol/l) and the number of 8-azaguanine resistant colonies was determined after 48 h of incubation. Survivors were scored by plating appropriate dilutions of conidia on CD-medium without 8-azaguanine.

Chinese hamster V79 cells were cultured in Dulbecco's minimum essential medium containing 10% fetal calf serum (Microbiological Associates), 100 IU/ml penicillin and 100 μg/ml streptomycin. The cells ($2·5–3 \times 10^6$ per plate, 5 cm diameter) were incubated in medium without serum containing 5 μg/ml (0·025% of ethanol was used as solvent for the drug) of the furocoumarin for 20 min in the dark at 37°C in a humidified 5% CO_2 atmosphere; irradiation was carried out as described above. The irradiated cells were detached from the dishes with 0·25% trypsin (1/250 Difco) and cell density was estimated by haemocytometer. For survival determination, 200 cells were plated on a 5-cm Petri dish (four plates for each dose). For mutant selection, 2×10^5 cells per 10-cm Petri dish were plated (five plates). Mutants defective in hypoxantine–guanine phosphoribosyl transferase (HGPRT) were selected by adding 6-thioguanine 1 h after plating at a final concentration of 4 μg/ml. Both survival and mutation plates were stained with 1% methylene blue 7 days after plating and colonies were scored macroscopically.

Clinical experiments

Four 6-methylangelicins, 6,4-DMA, 6,4′-DMA, 6,4,5′-TMA and the most active 6,4,4′-TMA, were tested for their effectiveness in clearing plaques of psoriasis in 17 patients (eight males and nine females) by their topical application and with UVA irradiation. 8-MOP was tested under the same experimental conditions.

Biopsies were taken 5 weeks later from the treated areas from six of the patients and processed for light microscopy. The patients gave their informed consent. They all had large plaque psoriasis for more than 1 year which was resistant to conventional treatment and to oral PUVA.

In the treated patients, four areas of 4 cm^2 of affected skin were used. In area 1, an ethanol solution (0·1 g% w/v) of the methylangelicin under investigation was applied at a concentration of 5 $\mu g/cm^2$, and left to dry. Forty-five minutes later the area was irradiated with high intensity UVA from a low-pressure mercury fluorescent lamp of the PUVA Waldmann Sylvania type. The irradiation doses were in the range 1·5–11 J/cm^2 and the initial dose was 1·5–2·5 J/cm^2 and was gradually increased to 5–11 J/cm^2. In area 2, an ethanol solution (0·1 g% w/v) of 8-methoxypsoralen (8-MOP) was applied at a concentration of 5 $\mu g/cm^2$ and the area was irradiated similarly to the first area. The third area was treated in the same way as the first, but was not irradiated with UVA. The last area was irradiated like the first with the same dose of UVA light, but without any compound and only the solvent being applied.

RESULTS

Toxicity

Toxicity in terms of LD_{50} was determined on mice for 6,4,4′-TMA only, because this compound appeared to be the most active one and was also the only one available in sufficient quantities. When administered orally to mice, this drug showed acute toxicity ($LD_{50} = 2$ g/kg), although much lower than that of 8-MOP when tested by oral administration ($LD_{50} = 0·75$ g/kg) and by i.p. injection ($LD_{50} = 0·3$ g/kg).

In vivo inhibition of epidermal DNA synthesis with topical and oral administration

The results of effects on epidermal DNA synthesis in mouse skin *in vivo* are shown in Table 1. Data obtained with 8-MOP are reported for comparison.

TABLE 1. Inhibition of epidermal DNA synthesis in mouse skin *in vivo* by angelicin methylderivatives after topical application and oral administration, followed by UVA irradiation (six animals were used for each experiment)

	% Inhibition ± SE	
Furocoumarin	Topical	Oral
8-MOP	61·0 ± 1·6	39·1 ± 3·7
6,4-DMA	47·0 ± 6·1	58·4 ± 9·0
6,4′-DMA	36·6 ± 5·6	49·2 ± 8·0
6,4,4′-TMA	73·7 ± 3·6	67·7 ± 8·2
6,4,5′-TMA	39·6 ± 5·2	36·8 ± 8·5

The most active compound appeared to be 6,4,4′-TMA, which was able to induce a marked inhibition of epidermal DNA synthesis of 73% and 67% when given topically and orally. 6,4-DMA also showed evident activity, while the effects of the other compounds were lower, although still evident. For some compounds, marked differences were seen between the effects of topical and oral administration. These may be explained in terms of pharmacokinetics, since different rates of absorption and/or excretion may occur with different routes of administration.

Photomutagenicity

The results obtained with 6,4′-DMA, 6,4,4′-TMA and 8-MOP on *Escherichia coli* WP2 uvrA are shown in Figure 2. Although the methylangelicins appeared to be much more cytotoxic than

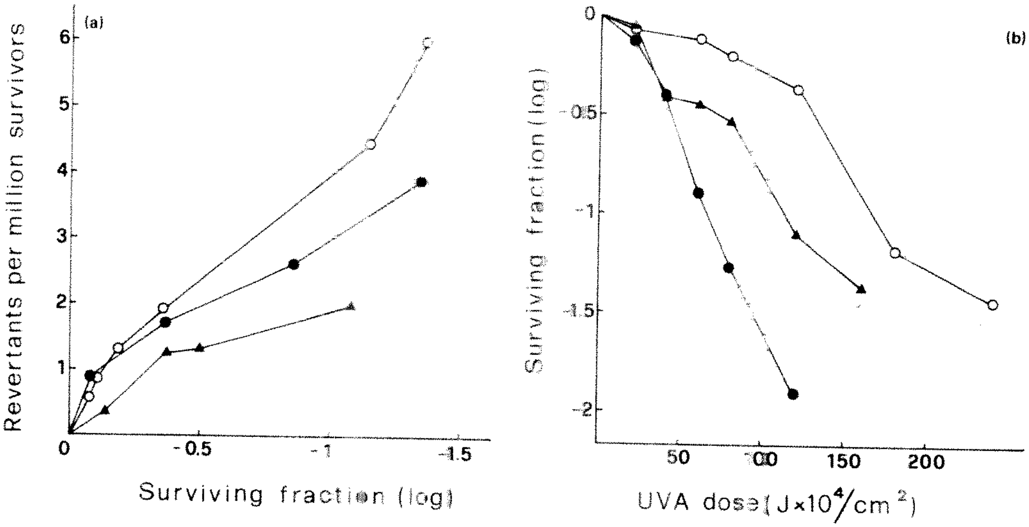

FIGURE 2. Mutagenic activity (a) and survival (b) in *E. coli* WP2 uvrA irradiated in presence of 6,4′-DMA (●) and 6,4,4′-TMA (▲) with 8-MOP (○) as reference compound. Points represent mean values from three different experiments; SE fall in following ranges: 0·4–0·15 revertants/10^6 survivors for mutagenesis and 0·1–0·2 log units for survival.

8-MOP (Fig. 2b), the latter showed higher mutagenicity when mutant induction was considered as a function of the surviving fraction (Fig. 2a).

Figure 3 shows the data obtained with *Aspergillus nidulans*. The methylangelicins seemed to be much less active than 8-MOP in inducing both lethality and mutagenicity in this micro-organism. However, in Chinese hamster V79 cells both 6,4′-DMA and 6,4,4′-TMA, but especially the latter, induced a more pronounced lethal effect than 8-MOP. However, when the surviving fraction was plotted against the mutant number, the three compounds gave practically overlapping curves (Fig. 4).

Clinical findings

No improvement was observed in the area treated but not irradiated while only a slight improvement occurred in the areas irradiated but without treatment with the compounds.

Table 2 shows the data for the areas treated with the four 6-methylangelicins and 8-MOP and with UVA irradiation. With 6,4,4′-TMA (patients 10–17) the clearance of the plaques was excellent in patients 10, 13, 14, 15, 16 and 17, and good in patients 11 and 12 (Fig. 5). Results using 8-MOP under the same conditions were good. The clearance using 6,4-DMA was excellent in patients 1, 2 and 4 and good in patient 3. In the case of 6,4′-DMA, improvement was excellent in patient 5 and good in 6. The corresponding effect induced by 8-MOP was good and only in patient 6 was it moderate. In the case of 6,4,5′-TMA, clearance was excellent in patient 9 and good in patients 7 and 8, and with 8-MOP it was good in all patients. It appears that 6,4,4′-TMA was more active than 6,4-DMA and 6,4′-DMA which, in turn, was more effective than 6,4,5′-TMA. However, all four 6-methylangelicins appeared to be more effective than 8-MOP.

The 6-month follow-up of the 17 patients showed a slight tendency towards recurrence of the

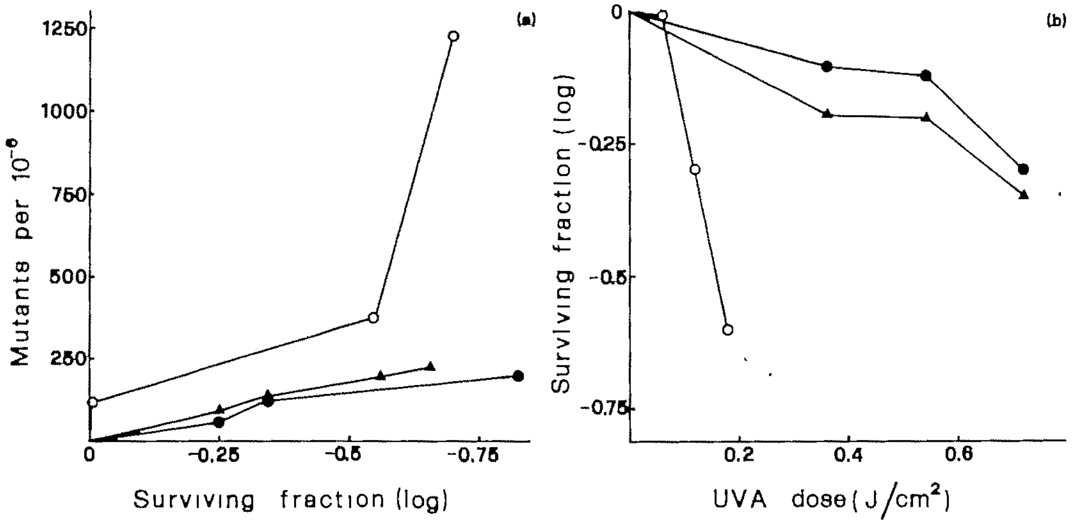

FIGURE 3. Mutagenic activity (a) and survival (b) in *Aspergillus nidulans* conidia by UVA irradiation in presence of 6,4′-DMA (●) and 6,4,4′-TMA (▲) with 8-MOP (○) as reference compound. Points represent mean values from at least three independent experiments; SE fall in following ranges: 0·6–0·2 mutants/10^6 survivors for mutagenesis and 0·1–0·2 log units for survival.

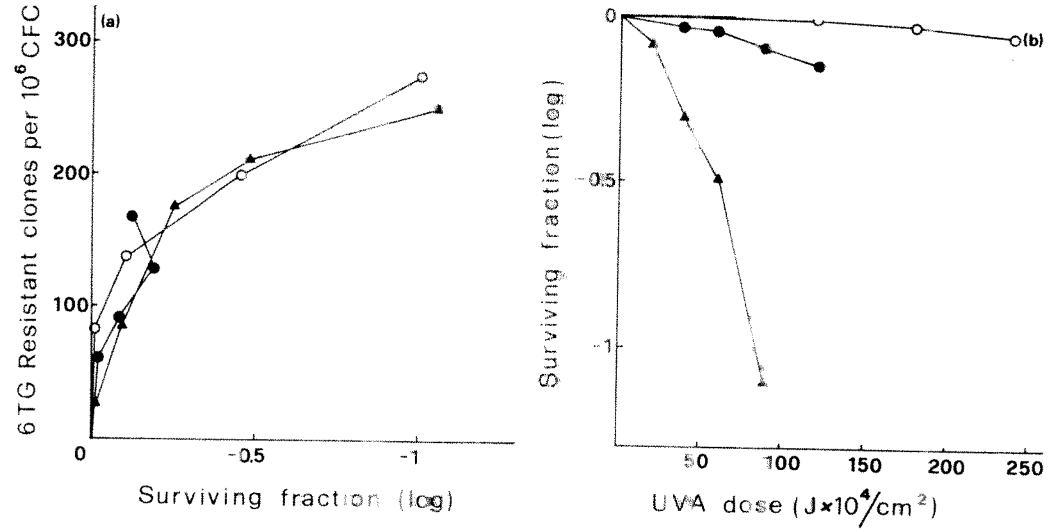

FIGURE 4. Mutagenic activity (a) and survival (b) in Chinese hamster V79 cells by UVA irradiation in presence of 6,4'-DMA (●) and 6,4,4'-TMA (▲), with 8-MOP (○) as reference compound. Points represent mean values from at least three independent experiments; SE fall in following ranges: 0·6–18% and 0·42–20% of mean value for mutagenesis and survival, respectively.

treated lesions. In patients 1, 3 and 11, the treatment with 8-MOP was discontinued after 4 or 5 applications because of blistering phototoxic reactions.

Histological findings

The results are based on the treatment of six patients. Common findings in areas treated with 8-MOP + UVA and 6,4,4'-TMA + UVA were: marked orthohyperkeratosis, mostly lamellar, keratotic plugs, hypergranulosis, and flattening of rete ridges. Biopsies from areas treated with 8-MOP showed foci of parakeratosis with neutrophils probably due to phototoxicity.

There was no evidence of disorganization of the basal-cell layer and the keratinocytes were large with an increased amount of pigment. Dyskeratotic cells were rarely found but especially so in the specimens treated with 8-MOP. The mitotic rate was normal. The melanocytes appeared to have increased in number.

Skin phototoxicity and pigmentogenic activity

Skin phototoxicity was evaluated on guinea-pig skin according to the method of Bordin *et al.*[19] In these conditions 6,4-DMA, 6,4'-DMA, 6,4,5'-TMA and 6,4,4'-TMA did not show any erythemogenic action even at high concentration of the drug (50 μg/cm²) and with a higher radiation dose (1·98 J/cm²). However, 8-MOP induced strong erythema even when small amounts of the drug were applied to the skin (5 μg/cm²) using a small dose (0·53 J/cm²) of UVA. These observations were confirmed on human skin and all the angelicins tested lacked skin photosensitizing activity. Only one patient treated with 6,4-DMA showed slight erythema; and in the other 16 patients no skin phototoxicity was observed. However, pigmentation of the skin was noted in the areas treated with the four 6-methylangelicins.

TABLE 2. Results of topical photochemotherapy with 6-methylangelicins in psoriatic patients

No. of Patient	Sex of patient	Angelicin	Cumulative UVA dose J/cm^2	No. of treatments	Clearance		Induction of erythema		Pigmentation capacity	
					Angelicin	8-MOP	Angelicin	8-MOP	Angelicin	8-MOP
1	M	6,4-DMA	78	20	Excellent	n.v.*	0	+ + +	+ +	n.v.
2	M	6,4-DMA	46	14	Excellent	Good	0	+ + +	+ +	+ +
3	M	6,4-DMA	8	4	Good	n.v.†	+	+ + +	+	n.v.
4	F	6,4-DMA	162	30	Excellent	Good	0	+ +	+ +	+ +
5	M	6,4′-DMA	54	15	Excellent	Good	0	+	+	+ +
6	F	6,4′-DMA	87	15	Good	Moderate	0	+	+	+ +
7	M	6,4,5′-TMA	63	21	Good	Good	0	+ +	+ +	+
8	M	6,4,5′-TMA	51	12	Good	Good	0	+ +	+ +	0
9	M	6,4,5′-TMA	64	13	Excellent	Good	0	+ +	+	+ +
10	F	6,4,4′-TMA	52	11	Excellent	Moderate	0	+ +	+ + +	+
11	F	6,4,4′-TMA	35	11	Good	n.v.†	0	+ + +	+ +	n.v.
12	F	6,4,4′-TMA	90	15	Good	Good	0	+ + +	+ +	+
13	F	6,4,4′-TMA	36	9	Excellent	Good	0	+ + +	+	+
14	F	6,4,4′-TMA	35	9	Excellent	Good	0	+ +	+	+
15	F	6,4,4′-TMA	112	18	Excellent	Good	0	+ +	+ +	+ +
16	M	6,4,4′-TMA	54	10	Excellent	Good	0	+ + +	+	+ +
17	F	6,4,4′-TMA	106	15	Excellent	Good	0	+ + +	+ +	+

*Treatment with 8-MOP was stopped after four applications, due to severe bullous reaction.

†Treatment with 8-MOP was stopped after five applications, due to severe bullous reaction.

% clearance: Excellent (100%) complete remission; good (75%); moderate (50%). Induction of erythema: 0, no erythema; +, barely perceptible; + +, pink; + + +, red, with oedema; + + + +, strong erythema.

Pigmentation capacity: + + +, Very strong; + +, strong; +, moderate; 0, absent.

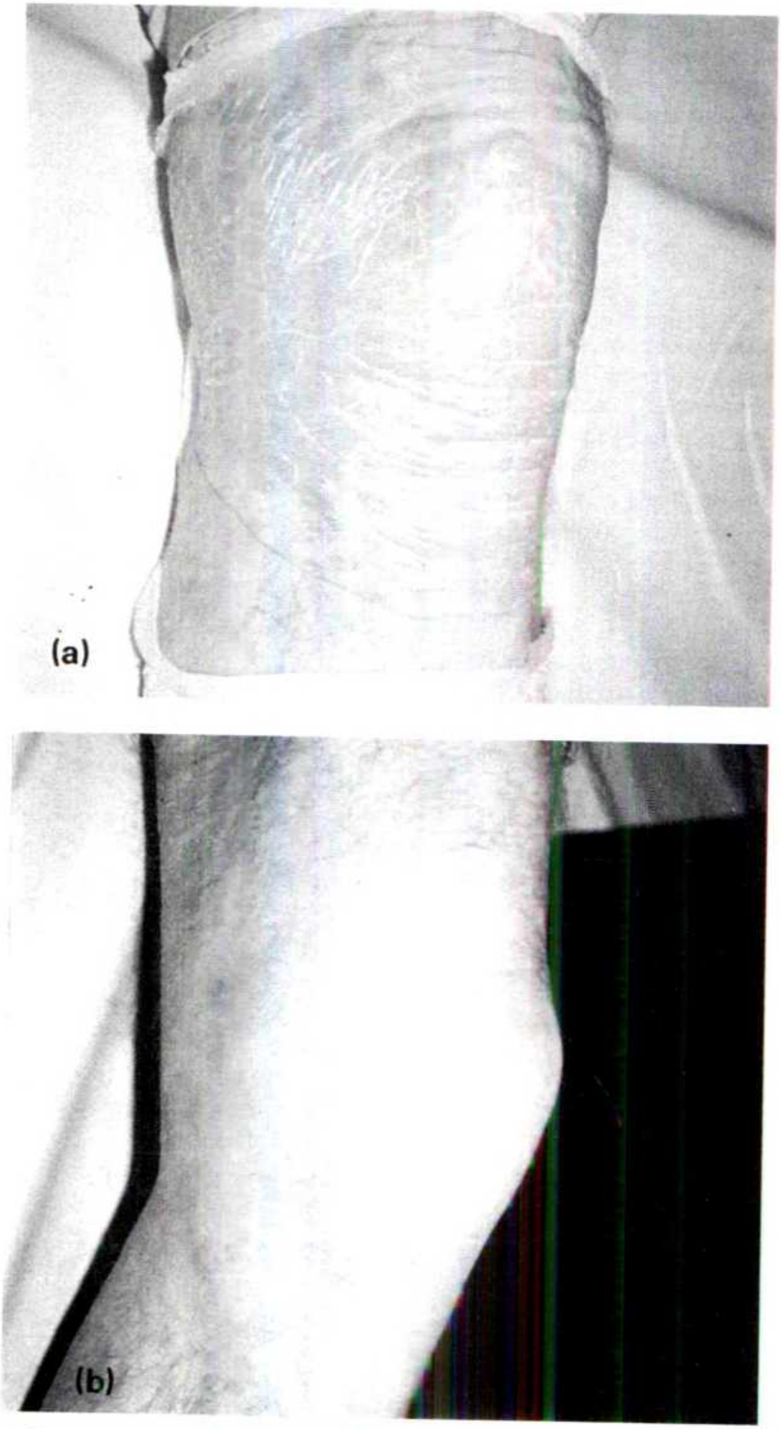

FIGURE 5. Chronic resistant lesions on a knee, before (a) and after (b) treatment with 6,4,4′-TMA.

DISCUSSION

The four new 6-methylangelicins examined showed marked photosensitizing activity. They showed marked inhibition of epidermal DNA synthesis in the mouse, when given both topically and orally and the most active compound was 6,4,4′-TMA.

In the photomutagenicity studies, 6,4′-DMA and 6,4,4′-TMA appeared to be less genotoxic than 8-MOP in *E. coli* WP2 uvrA and *Aspergillus nidulans*, while in Chinese hamster V79 cells, at the same survival level, they showed similar mutagenicity. However, it should be observed that the same lethal effect was obtained at lower UVA doses than with 8-MOP.

Thus, these data, together with the results obtained from DNA synthesis studies, show that methylangelicins, and in particular 6,4,4′-TMA, are capable of inducing stronger antiproliferative effects than 8-MOP. Although the pathogenesis of psoriasis is unknown it is a hyperproliferative disorder of the epidermis and a drug capable of inducing an antiproliferative effect may therefore be effective against psoriasis.

The four new 6-methylangelicins examined, and in particular 6,4,4′-TMA, were effective in clearing psoriasis in 17 patients when applied topically and with UVA irradiation. These drugs appeared to be more active than 8-MOP. These compounds, particularly 6,4,4′-TMA, did not induce phototoxicity, thus allowing it to be topically applied and avoiding side-effects due to systemic administration.

The mutagenic activity of 6,4,4′-TMA and 6,4′-DMA on mammalian cells is similar to that of 8-MOP when tested at the same level of cytotoxicity. 6,4,4′-TMA may be used topically at very small doses and for brief periods, thus reducing the area of the skin involved and limiting the side-effects connected with systemic administration.

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In vivo tretinoin-induced changes in skin mechanical properties

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SUMMARY

Topical tretinoin has been reported as having anti-aging effects on photodamaged skin. The purpose of this study was to evaluate tretinoin-induced changes in the mechanical properties of the skin of 18 patients (aged 39 ± 8 years) after 4 months of treatment with topical 0·05% tretinoin on one forearm and a placebo base cream on the other. The biomechanical skin parameters investigated were elasticity, extensibility and hysteresis and data were normalized for skin thickness. A slight but non-significant increase of skin elasticity was detected in the tretinoin-treated sites using low-stressing forces ($1{\cdot}2 \times 10^4$ Nm^{-2}) and at higher loads ($3{\cdot}8 \times 10^4$ Nm^{-2}), the increase in skin elasticity was significant ($P < 0{\cdot}01$). This improved skin elasticity was dependent on the increased collagen resulting from topical tretinoin and the replacement of elastotic material. However, topical tretinoin treatment did not improve the responses mediated by elastic fibres.

Topical tretinoin has been used for many years in the treatment of acne and hyperkeratotic skin conditions. Although its mechanism of action has not been completely understood, many effects of retinoids on epithelial differentiation and proliferation have been reported both *in vitro* and *in vivo*.[1] Recently, topical retinoic acid was reported as having anti-aging effects on photodamaged skin.[2] When 0·05% tretinoin was compared with its vehicle, the changes in the tretinoin-treated areas consisted of epidermal hyperplasia with elimination of dysplasia and atypia, removal of actinic keratoses, a more uniform distribution of pigment and new collagen formation in the papillary dermis. Non-invasive bioengineering techniques used in the same study showed an increased blood flow and transepidermal water loss.

The investigation of the biomechanical properties of the skin allows a rapid and non-invasive way of studying the structural and chemical organisation of skin layers. Age-related changes in skin constituents induce modifications of its mechanical parameters. There is general agreement that skin elasticity decreases with age. A study of skin biomechanics and Young's modulus using a torque method reported decreased elasticity after the age of 30, associated with an increase in

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the viscoelastic properties, and that Young's modulus doubles with age.[3,4] A progressive loss of elastic recovery with aging was reported which could be explained by a degenerative change in the elastin network of the dermis.[5]

In this article the effects of topical tretinoin treatment on skin mechanical properties are studied by means of a non-invasive, *in vivo* technique, monitoring several biomechanical parameters of the skin. These studies were carried out to determine the changes in the elastic and collagen components of the dermis following treatment with this retinoid.

METHODS

Eighteen Caucasian subjects (aged 39 ± 8 years) were included in the study. Informed consent was obtained and over a period of 4 months the left forearm was treated daily with topical tretinoin 0·05% and a similar area on the right forearm with a placebo cream. The biomechanical parameters were measured by means of a suction device (Dermaflex, Cortex Technology, Hadsund, Denmark). The probe was applied to the skin surface using a double-adhesive tape ring which contained a 1-cm diameter cup where the vacuum was applied. Measurements were carried out on the flexor aspect of the extended forearm using two different vacuum forces (100 mbars and 300 mbars, $1{\cdot}2 \times 10^4\ \mathrm{Nm}^{-2}$ and $3{\cdot}8 \times 10^4\ \mathrm{Nm}^{-2}$ respectively). The ambient temperature and relative humidity were kept constant. The subjects rested for 30 min prior to these procedures. For each measurement, the skin underwent five stress/strain cycles each lasting 5 s. The parameters investigated were skin extensibility, elasticity and hysteresis.

When a force is applied at the surface of the skin there is an immediate displacement followed by a delayed phase (OI, IE, Fig. 1). When the stimulus is discontinued, the skin tends to return to its initial position. After a few cycles the skin extensibility increases and the skin takes longer to return to the original position. In the present study, hysteresis is measured as the difference in maximum extensibility between the fifth and the first cycle; resilient distension is the skin's residual deformation measured at the last cycle. Elasticity was computed from the following formula: (Extensibility − resilient distension) × 100/extensibility. Extensibility and hysteresis were expressed in mm, elasticity as a percentage.

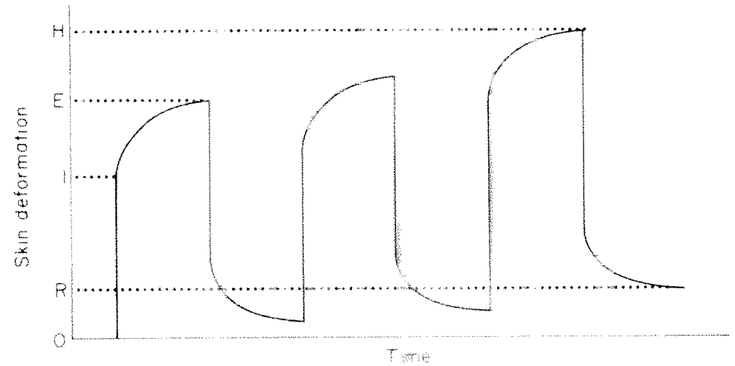

FIGURE 1. Skin deformation in relation to time. OI, immediate distension; IE, delayed distension; OR, resilient distension; EH, hysteresis.

TABLE 1. Skin thickness after treatment with tretinoin for 4 months. There are no significant differences

	Treated	Placebo
Average	0·88	0·92
Standard deviation	±0·13	±0·22

TABLE 2. No significant differences were detected after treatment with tretinoin when applying low loads

	Biomechanical parameters at 100 mbars (Average ± SD)		
	Treated	Placebo	P
Extensibility	1·64±0·35	1·57±0·32	NS
Elasticity	63·1±19	60·4±14	NS
Hysteresis	0·29±0·15	0·23±0·13	NS

TABLE 3. With higher loads a significant increase in skin elasticity was detected after tretinoin treatment (See also Figure 2)

	Biomechanical parameters at 300 mbars (Average ± SD)		
	Treated	Placebo	P
Extensibility	2·06±0·35	2·05±0·5	NS
Elasticity	66·0±12	58·9±13	<0·01
Hysteresis	0·32±0·12	0·26±0·08	NS

Statistical analysis of the data was performed by means of Student's *t*-test for paired data (two-tail).

RESULTS

No differences in skin thickness were detected between the tretinoin and placebo-treated sites (Table 1). When using lower loads, no significant differences in the biochemical parameters of the skin were found (Table 2). Extensibility and hysteresis increased respectively from 1·57 to 1·64 and from 0·23 to 0·29 mm (95% confidence intervals of the difference between −0·05 and

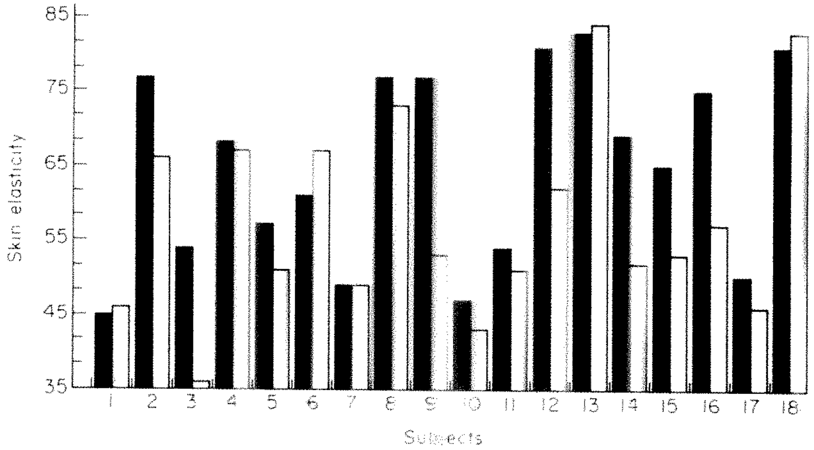

FIGURE 2. Skin elasticity at 300 mbars ($3{\cdot}8 \times 10^4\ \mathrm{Nm}^{-2}$): comparison between placebo-treated (placebo) and tretinoin-treated (treated) sites. The increase in skin elasticity on treated sites is significant (paired *t*-test 3·29, $P < 0{\cdot}01$). ■, tretinoin-treated skin; □, placebo-treated skin.

0·19 and between −0·02 and 0·14, respectively); elasticity increased from 60·4 to 63·1 (95% confidence intervals of the difference between −5·6 and 11·0).

At higher loads (Table 3), a significant increase in skin elasticity was detected ($P < 0{\cdot}01$). Values recorded were 58·9 in the placebo-treated site and 66·0 in the tretinoin treated site (Fig. 2) (95% confidence intervals of the difference between 2·55 and 11·6). Extensibility was unchanged, and hysteresis was slightly increased from 0·26 to 0·32 in the tretinoin-treated site (confidence intervals between −0·14 and 0·16, and between −0·01 and 0·1, respectively).

DISCUSSION

In this study topical treatment with tretinoin influenced skin elasticity, but not extensibility or hysteresis. Decreasing elasticity has been reported as the major mechanical parameter characterizing skin ageing.[3,5] Elasticity increased significantly ($P < 0{\cdot}01$) after 4 months of daily treatment with 0·05% topical tretinoin when compared with the placebo-treated site and when higher loads were used. No differences between the two sites were detected using lower forces. Low vacuum forces induce responses mainly by the upper dermis and elastic fibres;[5–9] in this phase, elastic fibres determine skin deformation and collagen fibres are realigned in the direction of the force without bearing the load. In an investigation of cutis laxa, a disease involving elastic fibres, changes in mechanical properties were found at a low stress with no tendency of the skin to return to its original shape. At a higher stress, the collagen bundles were involved and the mechanical properties became almost normal.[10] Thus, in this study the significant increase in skin elasticity after tretinoin treatment at high but not low loads implies an effect of tretinoin on the collagen network.

Elasticity, expressed as a percentage, is the time-dependent return to the initial position of the skin after deformation and takes into account both extensibility and resilient distension. Since extensibility did not show any significant changes, it is resilient distension (Fig. 1) of the skin that is the parameter which determines the increase in skin elasticity. This is due to an increase in newly formed collagen in the dermis, with improvement of dermal architecture and skin firmness. Indeed, several effects of this molecule on collagen metabolism and biosynthesis have been reported. Increased numbers of hyperactive fibroblasts with the formation of new collagen were found in hairless mice treated for 3 months with retinoic acid and there was replacement of elastotic material in the dermis.[11] These findings were confirmed in the guinea-pig.[12] Other effects include increased wound healing,[13] increased epidermal turnover and vascularization.[2] The increase in collagen in the dermis after treatment could be due to more synthesis or a decreased catabolism. Higher levels of hydroxyproline have been noted indicating more synthesis of collagen in granulation tissue,[14] and an inhibition of collagenase has been found with retinoic acid in fibroblast cultures.[15]

In this study, apart from elasticity, no other changes in mechanical parameters were detected. Extensibility has been reported to change with age but only after the seventh decade,[3,16] whereas the average age in our subjects was 39 years. Although an increase in dermal glycosaminoglycans was reported during treatment with retinoic acid in guinea-pigs,[12] no changes were recorded in the values of hysteresis, a parameter mostly dependent on the viscoelastic properties of the dermis and related to ground substance and glycosaminoglycan content.

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Serum lipid elevation during isotretinoin therapy for acne in the West of Scotland

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SUMMARY

We have studied 116 patients in the West of Scotland receiving isotretinoin for acne vulgaris at a dose of 1 mg/kg per day. No clinical evidence of adverse effects resulting from lipid elevations were seen in the short term. Cholesterol levels rose during the first 6 weeks of therapy, and thereafter stabilized, but triglyceride levels rose after 6 weeks therapy and continued to rise while therapy continued. Pretreatment levels of neither lipid was predictive of the level of elevation observed while on therapy. No other predictors of lipid elevation such as age or sex were identified in this study, which is the largest reported of patients receiving the standard recommended dose of isotretinoin.

The effect of oral isotretinoin on serum lipids is a source of concern. Several studies have found significant elevations in serum triglyceride and cholesterol concentrations during therapy.[1–6] However, the number of patients recruited in these studies have generally been small and the therapeutic regimes have varied, so that predictors for serious elevations in lipid values have not yet been established.

In the West of Scotland high levels of serum lipids in the population and a high incidence of cardiovascular disease are well recognized, and the consequences of isotretinoin therapy could be of greater importance to Scottish patients. We have therefore reviewed 116 patients with acne vulgaris given the standard regime of 1 mg isotretinoin per kg per day.[7] The overall increases in serum lipids were determined and predictors of high risk were sought.

METHODS

We studied 116 patients with severe nodulocystic acne treated during a 2-year period. There were 76 males and 40 females aged 15–49 years (mean 24 years). Each patient received 1 mg isotretinoin per kg per day (Roaccutane, Roche Laboratories) for periods from 4 to 26 weeks. Forty-six patients were treated for exactly 16 weeks.

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Prior to starting therapy, 10 ml of venous blood were taken after a 14-h fast and analysed by COBAS B10 Centrifugal analyser (Roche Diagnostic). Further samples were taken at varying intervals throughout the course of treatment: 56 patients had samples taken between 2 and 6 weeks after the start of treatment; 65 patients between 6 and 10 weeks; 40 patients between 10 and 14 weeks; and 36 patients beyond 14 weeks.

The normal range for the West of Scotland population is 3·1–6·5 mmol/l for cholesterol and less than 2·1 mmol/l for triglyceride. The treated group was a representative sample of this population with a pretreatment cholesterol mean of 4·93 ± 2·4 mmol/l and pretreatment triglyceride mean of 0·95 ± 0·96 mmol/l.

Statistics were performed on an IBM computer with the MINITAB program. To establish whether absolute increases (in mM/l) or relative increases (as percentages of pretreatment levels) in lipid levels were more appropriate, we plotted the absolute increase against pretreatment levels for each patient. There was no correlation between these, and all results are presented in terms of absolute increases.

RESULTS

Overall increases in serum lipids and influence of duration of therapy

There were statistically significant increases in serum lipids throughout the course of therapy (Table 1), the greater part of these increases occurring within the first 6 weeks of treatment. Table 1 also shows lipid levels in subsequent months compared with those in the first 6 weeks. For the population as a whole there was no further significant elevation in mean levels after 6 weeks. However, individual patients might have had clinically relevant lipid elevation at a later stage, and Figures 1 and 2 include all patients who had cholesterol or triglyceride levels greater than 2 standard deviations (SD) above the mean at any time during therapy, and for whom more

TABLE 1. Changes in serum lipids during therapy, and comparison of levels in subsequent weeks with dose after 2–6 weeks of treatment

	Cholesterol minus pretreatment cholesterol (mean ± SD) (mmol/l)	Triglyceride minus pretreatment triglyceride (mean ± SD) (mmol/l)	Cholesterol minus cholesterol at 2–6 weeks (mean ± SD) (mmol/l)	Triglyceride minus triglyceride at 2–6 weeks (mean ± SD) (mmol/l)
2–6 weeks of treatment	0·57 ± 0·59	0·43 ± 0·60	—	—
6–10 weeks of treatment	0·52 ± 0·68	0·45 ± 0·64	0·02 ± 0·49	0·06 ± 0·42
10–14 weeks of treatment	0·76 ± 0·9	0·62 ± 1·00	0·08 ± 0·84	0·35 ± 1·27
> 14 weeks of treatment	0·40 ± 0·8	0·58 ± 1·70	−0·37 ± 1·2	−0·28 ± 0·94
Maximum at any time of treatment	0·71 ± 0·7[illegible]	0·69 ± 1·21	—	—
	$P < 0·05$		$P > 0·1$	

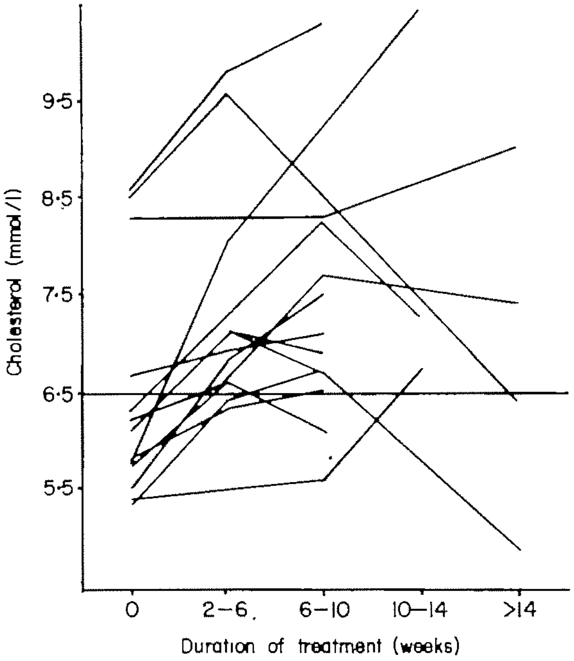

FIGURE 1. Serial serum cholesterol in those patients with cholesterol at any time ≥ 6 5 mmol/l.

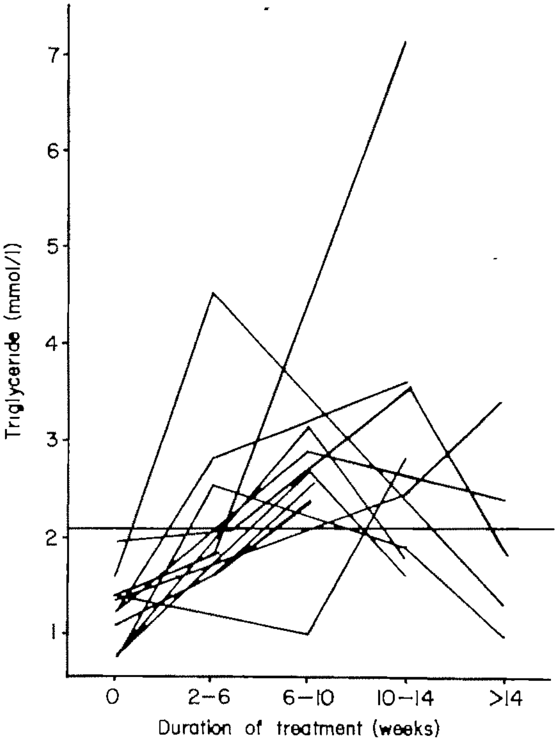

FIGURE 2. Serial serum triglyceride in those patients with triglyceride at any time ≥ 2 1 mmol/l.

TABLE 2. Influence of duration of therapy, age, sex and pretreatment lipid values on maximum elevations of lipids during therapy

	Weeks of treatment		Sex		Age (years)			cholesterol (mmol/l)		triglyceride (mmol/l)	
	> 16	16	Male	Female	15–20	21–30	31–50	< 6·5	> 6·5	< 2·1	> 2·1
Maximum elevation of cholesterol (mean ± SD) (mmol/l)	0·70 ± 0·97	0·74 ± 0·59	0·76 ± 0·79	0·60 ± 0·74	0·75 ± 0·66	0·57 ± 0·68	0·86 ± 1·12	0·71 ± 0·77	0·60 ± 0·73	—	—
Maximum elevation of triglyceride (mean ± SD) (mmol/l)	1·06 ± 1·80*	0·49 ± 0·72*	0·76 ± 1·39	0·54 ± 0·72	0·52 ± 0·72	0·69 ± 1·52	1·01 ± 1·40	—	—	0·68 ± 1·20	0·77 ± 1·75
Number of patients	40	45	76	40	50	43	22	110	6	113	3

By one way analysis of variance, none of these results approaches statistical significance except *, for which $F = 3{\cdot}84$ (F required $> 3{\cdot}9$ for $P < 0{\cdot}05$).

than one result during therapy was available. With cholesterol (Fig. 1), very few abnormal values were preceded by normal results in the first 6 weeks. This does not apply to triglyceride values where an upward trend was seen at this time (Fig. 2). The patient showing a dramatic rise of triglyceride had isotretinoin withdrawn and his triglycerides returned to the normal range within 4 weeks. No adverse clinical events occurred.

In Table 2 a group of patients treated for exactly 16 weeks is compared with those treated for longer than 16 weeks. The maximum cholesterol elevation was the same in both groups, but in the patients treated for a longer period there was a higher elevation of triglyceride which approached statistical significance.

Influence of age and sex on serum lipids increases

There was no relationship between maximum elevations of lipids and the age or sex of the patients (Table 2).

Patients with lipid concentrations of possible clinical significance

The literature suggests that there is a linear relationship between serum cholesterol level's and long-term cardiovascular complications,[8] but short-term complications of hypertriglyceridaemia have rarely been seen as a result of therapy with isotretinoin.[9,10] There are no guidelines for the level of cholesterol or triglyceride at which a review of retinoid dosage should be made. We therefore chose the upper limit of the normal range (2 SD) as an arbitrary level for concern, and analysed the demography and changes in serum levels for any patients with higher than normal values before or during therapy.

The results for these patients are summarized in Tables 3 and 4. Elevated pretreatment levels did not predict unusually high absolute elevations in lipid values during therapy. There was no trend in age or sex distribution or duration of therapy amongst patients who developed abnormal lipid levels during therapy (Table 3). There was no evidence of a relationship between abnormal cholesterol levels and abnormal triglyceride levels either before or during treatment

TABLE 3. Demography of patients attaining lipid levels during therapy which exceed the normal range (to 2 SD)

		Sex		Age (years) mean ± SD	Duration of treatment (weeks)	No of Patients
		Male	Female			
Maximum cholesterol level during therapy (mmol/l)	≥6·5	11	6	28·3±10·1	13·7±5·8	17
	<6·5	64	34	23·6±6·4	17·0±4·3	99
Maximum triglyceride level during therapy (mmol/l)	≥2·1	16	5	28·1±11·1	17·5±6·0	21
	<2·1	60	35	23·5±5·8	16·32±4·3	95

χ^2, not significant.
Student *t*-tests not significant.

TABLE 4. Relationships between cholesterol levels and triglyceride levels before and during therapy

	Numbers of patients			
	Triglyceride < 2·1 mmol/l		Triglyceride > 2·1 mmol/l	
	Pretreatment	On treatment	Pretreatment	On treatment
Cholesterol < 6·5 mmol/l	109	85	2	14
Cholesterol > 6·5 mmol/l	4	10	1	7

(Table 4). None of our patients developed clinical problems attributable to lipid elevation during isotretinoin therapy.

DISCUSSION

This study concerns the largest number of patients yet reported receiving the current recommended dose of isotretinoin for acne. The findings of increases in serum cholesterol and triglyceride levels are well established.[1-6] The results of this study concur with previous studies which document maximum mean increases of triglyceride from 4·8 mmol/l[2] to 1·3 mmol/l[3] and of cholesterol from 0·6 mmol/l[2] to 1·0 mmol/l.[3] The increase in triglyceride was reported to be dose dependent,[4] in the range 0·25–1·0 mg/kg per day but cholesterol levels were not quoted in this study. Dosage of isotretinoin and duration of therapy have varied in previous studies. Most concerned fewer than 20 subjects[1,3,5,6] and where larger numbers were included they were divided into different dosage groups of 21 patients.[4] For example, Vahlquist *et al.*[5] treated 12 patients for 8 weeks with 40 mg daily while Zech[1] treated 20 men for 4 months with 0·5 mg/kg per day. The only similar study with a large number of patients on a standard dosage, is that of Bershad *et al.*[2] who studied 60 patients receiving 1 mg/k per day for 20 weeks. Our results are similar in confirming only modest elevations in lipids, contrary to earlier reports.[11]

Bershad *et al.*[2] demonstrated a greater increase in triglycerides during the first month of therapy in men compared with women. In contrast, it was found that there was no difference between the sexes in maximum increases in lipids or in the patients who developed levels higher than the normal range. Increases in cholesterol took place within the first 6 weeks and thereafter there was no progressive elevation. There was no apparent relationship between the duration of therapy and the maximum elevation in cholesterol and the level of cholesterol measured at 6 weeks appears to be a good predictor of subsequent unusually high levels (Fig. 1). There was no evidence that patients with unusually high levels of cholesterol had received longer courses of treatment.

While Table 1 does not show progressive elevation of triglycerides, Table 2 suggests that the maximum increase in triglyceride may be dependent on duration of therapy, although this did not reach statistical significance. The level of triglyceride at 6 weeks was a poor predictor of unusually high levels of triglyceride in subsequent months (Fig 2), and re-examining the data of Bershad *et al.*[2] and Zech *et al.*[1] shows that while the cholesterol elevations were well established within the first 6 weeks, those of triglyceride occurred later, and in a more progressive manner.

It remains to be established whether the degree of elevation of cholesterol associated with retinoid therapy is of any clinical significance, particularly as it returns to normal when treatment is discontinued.[12] However, episodes of acute complications of hypertriglyceridaemia have occurred.[9,10] The first report concerned a patient treated for Darier's disease, but the second was a young woman treated for acne for only 7 weeks, and at a normal dose of 1 mg/kg.

No identifiable risk factors were found for lipid elevation such as age or sex, nor that pretreatment levels predict a risk of extreme elevation in serum lipids. Levels of triglyceride and cholesterol should be measured before and within 6 weeks of retinoid therapy. If no dramatic increase in cholesterol is observed then, this assay need not be repeated. However, with triglyceride there is the possibility of a continuing increase in serum levels, and immediate complications may be more threatening than for cholesterol. Monthly measurement of triglyceride levels is therefore advised. Currently being studied is the value of an instant lipid analyser, as results available within 10 min of venepuncture offer the opportunity for instant dietary advice and alteration in dosage, thus reducing the number of hospital visits required for a course of standard therapy.

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Effect of BN 52021, a platelet activating factor antagonist, on dithranol-induced inflammation

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SUMMARY

The effect of BN 52021, a selective platelet activating factor (PAF) antagonist was studied on dithranol-induced irritant dermatitis. Pretreatment of the skin with 0·45% BN 52021 ointment significantly suppressed the decrease in capillary resistance, rise in skin temperature and increase in skin-fold thickness produced by dithranol.

Dithranol is highly effective in the treatment of psoriasis, but its use has been limited by its irritancy. It is difficult to reduce the irritant effect with the commonly used anti-inflammatory agents,[1–3] and although several studies suggest that free radicals are involved,[4–6] the mechanism of the irritancy is still not fully understood.

Dithranol-induced dermatitis is characterized by an increased vascular permeability, plasma protein exudation and the immigration of inflammatory cells into the skin.[6,7] All these can be produced by the potent, pro-inflammatory phospholipid, platelet activating factor (PAF).[8] Recently, indirect evidence of the involvement of PAF in dithranol dermatitis in mice was found through the use of a highly specific PAF antagonist, BN 52021.[9] This ginkgolide B compound has been shown to antagonize many, if not all of the biological activities induced by PAF.[10]

In this study the effect of locally applied BN 52021 ointment was tested on dithranol-induced dermatitis in healthy individuals. The extent of the inflammation was determined by a decrease in skin capillary resistance (CR), a rise in skin temperature and an increase in skin-fold thickness produced by the dithranol.

METHODS

Ethical Committee approval and informed consent was obtained and 33 healthy volunteers (19 males and 14 females; age range 21–45 years, mean age 35·5 years) were included in the study.

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The subjects had skin types II–III, and had not been on any treatment during the previous 3 months.

Changes in skin capillary resistance induced by dithranol, and the effects of BN 52021

Dithranol inflammation was induced on the scapular regions of the back of seven healthy individuals, as described previously.[11] Briefly, 0·80 g of 0·25% dithranol (Bayer AG, Leverkusen, F.R.G.) in an emulsifying ointment and containing 3% salicyclic acid was applied to a 100 cm^2 area of the clinically normal skin of the back for 1 h. The dithranol ointment was always freshly prepared. The right side was pretreated with 0·45% BN 52021 ointment (Institut Henri Beaufour, Le Plessis Robinson, France) 6 h prior to the elicitation of the dermatitis, while the left scapular region was pretreated with the ointment base.

The capillary resistance (CR) in the dithranol-treated areas was measured with a Parrot angiosterrometer before and at different times after the challenge with the drug. The CR was designated as the minimum suction value (expressed in mmHg) at which the first petechia appeared after application of the suction cup (2 cm diameter) for 1 min.[12]

Changes in skin temperature and skin-fold thickness induced by dithranol, and the effects of BN 52021

Inflammation was induced on the clinically normal volar forearm skin of 26 healthy volunteers, as previously described.[2] Briefly, 10, 20, 40, 80 or 100 μg dithranol dissolved in 10 μl chloroform was applied to the skin inside a plastic well 15 mm in diameter and the chloroform allowed to evaporate off. The test sites were marked and left uncovered without washing for at least 12 h. The right forearm was treated with 0·45% BN 52021 ointment 2, 6 or 12 h prior to, immediately after or 18 h after the elicitation of the dermatitis, and a corresponding control site on the opposite arm was treated with the ointment base.

The inflammatory reaction to dithranol was characterized by the rise in skin temperature[13] and the increase in skin-fold thickness.[14] Skin temperature measurements were made with a contact thermometer 48 h after the elicitation of the dermatitis. Three readings were made at each test site, one at the centre of the inflamed area and two others 2 cm lateral to the dithranol staining. The difference between the reading at the centre of the test site and the average of the control readings was taken as the rise in skin temperature. At each test site, the skin-fold thickness was measured with Harpenden skin calipers immediately before and 48 h after the elicitation of the dermatitis.

Statistical analysis

The results were analysed by paired Student's *t*-test or by analysis of variance.

RESULTS

Effects of BN 52021 ointment on the dithranol-induced decrease in capillary resistance

Dithranol caused a significant decrease in the CR in the control base-pretreated skin. This decrease reached its maximum during the first hour and was then reversed (Fig. 1). Pretreatment of the skin with 0·45% BN 52021 ointment 6 h before elicitation of the dermatitis significantly moderated the CR-lowering effect of dithranol. Six hours after the challenge, the CR had already returned to the normal level in the BN 52021-pretreated skin, whereas the CR became normal only 12 h after dithranol treatment on the ointment base-pretreated side.

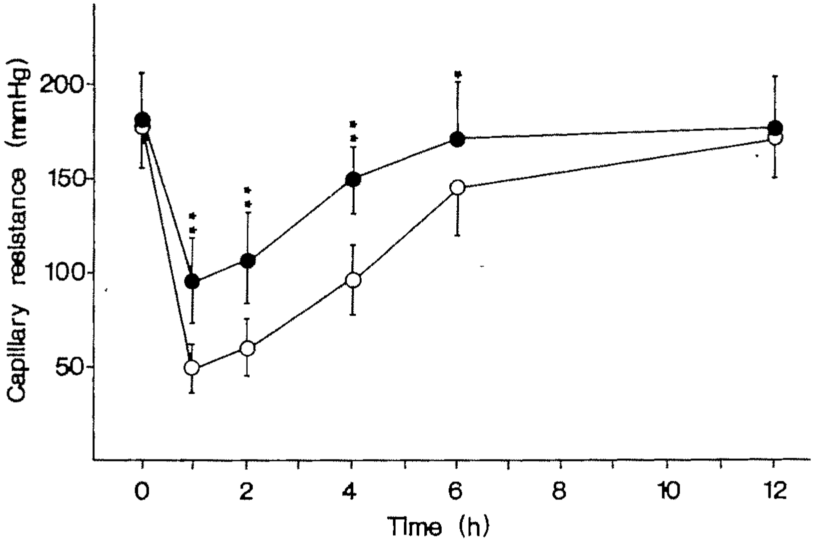

FIGURE 1. Effects of a 6-h pretreatment with 0·45% BN 52021 ointment (●) or with the ointment base (○) on the 0·25% dithranol-induced decrease in capillary resistance measured before and at different times after the challenge. * = $P < 0{\cdot}05$, ** = $P < 0{\cdot}01$ (Student's paired *t*-test). Figures show means ± SD ($n = 7$).

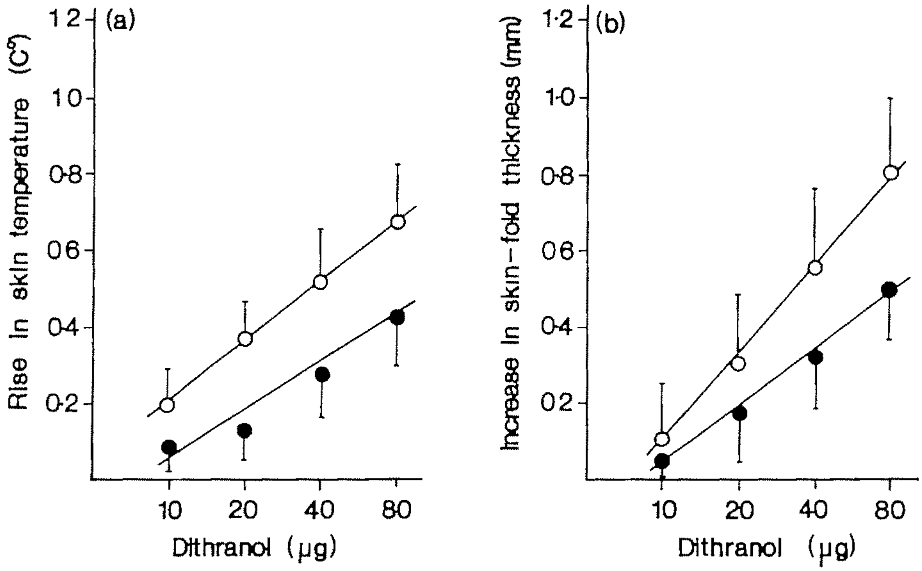

FIGURE 2. Effects of a 6-h pretreatment with 0·45% BN 52021 ointment (●) or with the ointment base (○) on the dithranol-induced rise in skin temperature (a) and increase in skin-fold thickness (b) measured 48 h after the challenge. Comparison of the full dose–response curve by analysis of variance, $P < 0{\cdot}05$. Figures show means ± SD ($n = 10$).

Effects of BN 52021 ointment on the dithranol-induced rise in skin temperature and increase in skin-fold thickness when applied prior to the challenge

Dithranol caused a consistent and significant dose-dependent rise in skin temperature and increase in skin-fold thickness. Pretreatment of the skin with 0·45% BN 52021 ointment 6 h prior to the challenge significantly inhibited both the rise in skin temperature and the increase in skin-fold thickness induced by 10, 20, 40 and 80 μg dithranol (Fig. 2).

BN 52021 ointment had a small, but significant effect on the dermatitis when applied 2 h before dethranol treatment. The effect was greater when it was used 6 or 12 h prior to the challenge (Fig. 3), with no significant difference between these two pretreatment times.

Effects of BN 52021 ointment on the dithranol-induced rise in skin temperature and increase in skin-fold thickness when applied after the challenge

BN 52021 ointment slightly reduced the dermatitis when applied just after dithranol treatment, and had no effect when applied 18 h after the challenge (Fig. 4).

DISCUSSION

Pretreatment of the skin with BN 52021 ointment 6 h before induction of inflammation by dithranol significantly suppressed the decrease in the CR, the rise in skin temperature and the increase in skin-fold thickness. The inhibitory effect of the drug was less pronounced but still significant when it was applied 2 h before or immediately after the challenge, but it had no effect on inflammation when applied 18 h after the dithranol treatment from the time of onset of erythema.

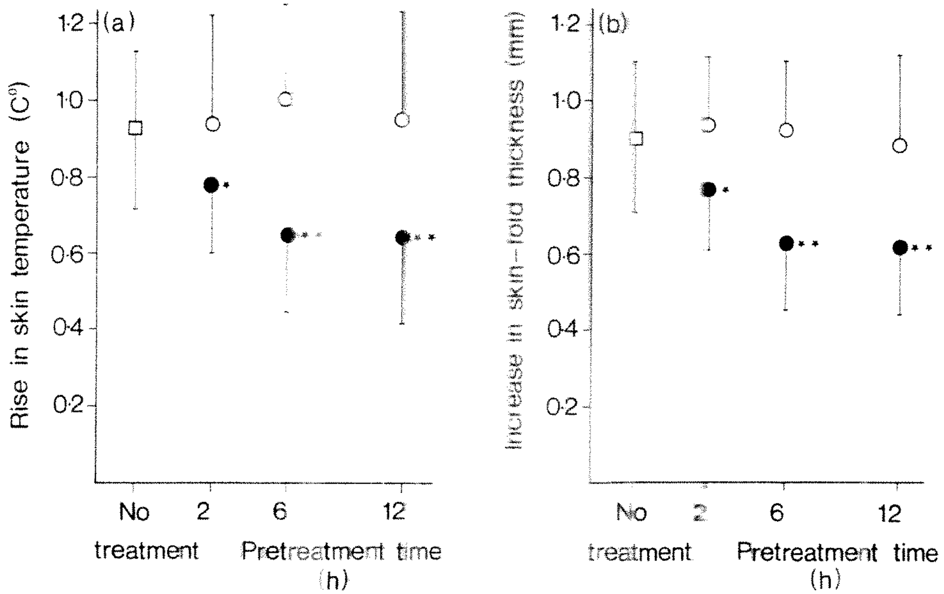

FIGURE 3. Effects of 0·45% BN 52021 ointment (●) or the ointment base (○), applied 2, 6 or 12 h prior to the challenge, on the 100 μg dithranol-induced rise in skin temperature (a) and increase in skin-fold thickness (b). (□) = without pretreatment, * = $P < 0{\cdot}05$, ** = $P < 0{\cdot}01$ (Student's paired *t*-test). Figures show means ± SD ($n = 8$).

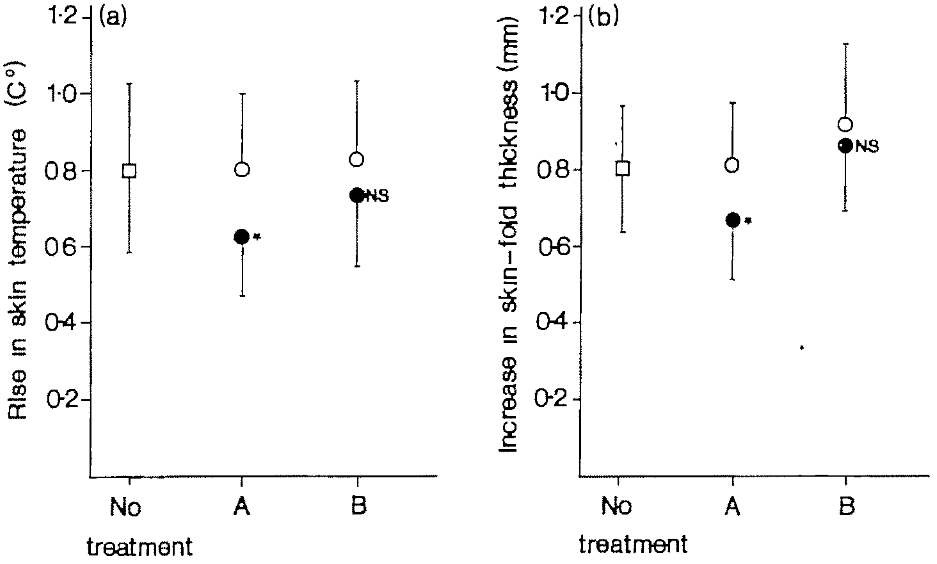

FIGURE 4. Effects of 0·45% BN 52021 ointment (●) or the ointment base (○) applied immediately (A) or 18 h after (B) the application of dithranol, on the 100 μg dithranol-induced rise in skin temperature (a) and increase in skin-fold thickness (b). □, without treatment. $* = P < 0{\cdot}05$; NS, not significant (Student's paired *t*-test). Figures show means ± SD ($n = 8$).

BN 52021 is a highly effective PAF antagonist, which has been reported to selectively anatagonize many PAF activities both *in vivo* and *in vitro*.[10] Dithranol-induced dermatitis is thought to be due to tissue oedema and the immigration of inflammatory cells into the skin.[7] Intradermal injection of PAF induces microvascular leakage and accumulation of inflammatory cells in human skin.[15] Since the PAF antagonist BN 52021 moderated the dithranol dermatitis, we conclude that PAF is involved in dithranol irritancy in humans.

The minor effect of BN 52021 when applied 2 h before treatment with dithranol may be due to incomplete penetration into the skin. Since the decrease in CR reached its maximum 1 h after elicitation of the dermatitis, and pretreatment of the skin with BN 52021 ointment suppressed this effect, we suggest that PAF is involved in the early stage of this dermatitis. The BN 52021 ointment had no effect on the dithranol-induced rise in skin temperature and increase in skin-fold thickness when applied 18 h after the challenge, and its inhibitory effects was less marked when it was applied immediately after dithranol treatment.

Recently, indirect evidence was provided of the involvement of PAF in dithranol[9] and croton oil-induced irritant dermatitis and in allergic contact dermatitis[16] in mice. The source of PAF in these inflammatory skin conditions has not yet been clarified. It was found that murine epidermal cells show an indomethacin-insensitive platelet-aggregating activity after calcium ionophore A 23187 stimulation.[17] Apart from epidermal cells, a wide range of cells of different lineages have been reported to be capable of producing PAF *in vitro* when they are stimulated under various conditions.[18] This has been shown to occur in the inflammation of the skin of guinea-pigs irradiated with UV light.[19]

Many different mediators, such as free radicals,[4] histamine[11] and prostaglandins,[13] have previously been suggested as playing a role in dithranol-induced irritancy in humans. This study provides indirect evidence of the involvement of PAF, a highly potent pro-inflammatory mediator in the development of the dermatitis.

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The antipruritic effect of a sedative and a non-sedative antihistamine in atopic dermatitis

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SUMMARY

A double-blind, randomized, cross-over study was carried out on the effect of a sedative and a non-sedative antihistamine on 25 adults with atopic dermatitis. Intensity of itch was recorded using a computerized method for self-assessment (Pain-Track®) and using conventional visual analogue scales. The antipruritic effect of 3 days of treatment with the non-sedative H_1 antagonist terfenadine (60 mg b.i.d.) and with the sedative antihistamine, clemastine (2 mg b.i.d.) did not differ from that found with the placebo. Our findings support the view that histamine is not of importance in the pathogenesis of itch in atopic dermatitis.

Although itching is a major feature in atopic dermatitis (AD),[1] its pathogenesis remains unknown. The traditional H_1-receptor antagonists are widely prescribed as antipruritics in various non-wealing dermatoses, including eczema. In an open trial of 23 patients with ‘eczematous dermatosis’ or psoriasis it was concluded that the antipruritic effect of antihistamines was due to a central, sedative-related effect and not to peripheral H_1 antagonism.[2] In a placebo-controlled cross-over study of 10 adults with chronic AD, a new potent non-sedative H_1 antagonist LN 2974 failed to reduce itching and scratching.[3] However, an antipruritic effect was found after 10 days’ treatment with either acrivastine or terfenadine, non-sedative antihistamines, when compared with placebo in a double-blind, but parallel, study in 44 adults with AD.[4] Thus, contradictory results have been reported concerning the antipruritic effect of non-sedative antihistamines.

The aim of this study was to use one of the new non-sedative antihistamines to determine the role of histamine in the pathogenesis of pruritus in AD. The effects of the non-sedative H_1-receptor antagonist terfenadine were compared with those of the sedative antihistamine clemastine and placebo.

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METHODS

Subjects

Twenty-five adults (nine males and 16 females; median age 24 years, age range 17–42 years) with persistent AD according to the criteria of Hanifin and Rajka[3] participated in the study, having given informed consent. At the initial clinical examination they were asked about their symptoms. All had chronic pruritus for at least the previous year, and had attended our department over the previous 6 months. Patients with AD limited to the face or hands were excluded. Other exclusion criteria were the taking of any drug, pregnancy or lactation, skin infections, and the presence of urticaria or other skin diseases. The study was performed by the same investigators in the period December–March, and was approved by our Ethics Committee.

Experimental design

The course of treatment was terfenadine 60 mg b.i.d., clemastine 2 mg b.i.d. and placebo each for 3 days and with intervening washout periods of 4 days and according to a randomized, double-blind, 'double-dummy', cross-over protocol. Thus, each patient received three courses in random order: (i) terfenadine active + clemastine placebo, (ii) terfenadine placebo + clemastine active and (iii) terfenadine placebo + clemastine placebo. The subjects were not allowed any other oral medication or alcohol, and were instructed not to apply any topical corticosteroid more potent than 1% hydrocortisone in the week before and during the whole study. None of the patients had ultraviolet light therapy during the previous month. The use of emollients, however, was permitted. On days without oral treatment, a hydrocortisone cream was permitted and the amount used was determined. On the third day of each course of medication, tests for histamine-induced skin flare and itch reactions were performed.

Experimental flare and itch

An experimental flare and itch was induced 5–7·5 h after the ingestion of the morning tablet. Histamine solution (0·01 ml) (100 µg/ml) and a similar amount of buffered saline as control were injected intradermally in duplicate on the outer aspect of the upper arm. The injection sites were gradually changed to avoid tachyphylaxis to histamine.[5] The flare reaction 5 min after injection was outlined and traced onto clear plastic film and the area measured planimetrically (mm^2). The intensity of the histamine-induced itch was recorded over a period of 15 min by the subjects, using an indicator sliding along a 100-mm visual analogue scale (VAS). One end of the scale represented 'no itch' and the other 'maximal itch'. The indicator was attached to a potentiometer connected to a plotter out of sight of the patient. The recordings allowed the calculation of itch duration (ID, s), peak value of itch (Imax, 0–100 mm) and a 'total itch index' (Tii = area under the curve, mm^2) as described elsewhere.[6]

Clinical itch and sedation

On the last day of each treatment period the patients rated the total intensity of itch and sedation for that time using 100-mm VAS on sheets of paper. The results were collected before the experimental skin test and sealed immediately. During the 24 h before and in the period of 17 days of the study the itch intensity was monitored continuously using the Pain-Track® computerized method.[7]

Pain-Track®

Pain-Track® (Autenta AB, Uppsala, Sweden) is a system for the self-recording of subjective symptoms.[6,7] It consists of portable data-loggers for patients' use, a terminal unit and a software package for storage, analysis and presentation of data. The logger has a switch for indicating awake/asleep, a button for marking the patient's presence, and a knob for rating itch intensity on a fixed-point non-verbal scale (FPNVS) from 0 ('no itch') to 6 ('maximal itch'). When the switch is in the 'awake' position a buzz every 60 min requests the subject to press the marker button and to rate the intensity of pruritus at that moment. The knob can also be turned at any time, and during the night the switch is turned to the 'sleep' position to cut out the hourly buzz. The setting of the controls is recorded by the computer at sampling intervals of 10 min. Compliance is calculated as the percentage of the patient's responses to the buzzer. All days with a compliance of less than 70% were excluded. The results from days 2 to 3 were used, as a pilot study showed that a steady-state for the inhibition of histamine-induced skin flare had been reached during these days. The 'itch variables' analysed were the median and average itch intensity based on samples every 10 min and the percentage of time awake without itch. The 'itch profile' was based on samples every hour.

Statistical analysis

For the experimental skin test results the non-parametric Wilcoxon signed-rank test was used, as normal distribution of these data was considered doubtful. For clinical itch, sedation and hydrocortisone consumption, analysis of variance was used, as the assumptions of interval-scale basis and normal distribution were considered reasonable for these variables. Paired follow-up analyses were performed using the standard t-test as the number of comparisons was small. As a check on the assumptions, non-parametric tests were also performed. The test used was one based on m-rankings with Wilcoxon scores, applicable to unbalanced factorial designs, which in balanced situations reduces to the Friedman non-parametric test. A test was carried out on the 'itch profile' using an analysis of variance model with the factors treatment, time and subject. The pattern of itch within the day was further analysed using an orthogonal polynomial decomposition.

RESULTS

Data from introductory patient interviews

In 13/25 patients (52%) the AD started before 1 year of age (range 6 weeks to 37 years). The median duration of eczema with itching was 20 years (range 2–39 years). The majority, 20/25 (80%), also had a history of asthma/hay fever, but no one was on any treatment for these conditions. In 21/25 (84%), the most pruritic period was generally in the evenings or at night and only 3/25 (12%) reported the worst itching in the morning. Itch disturbed the sleep of 22/25 (88%) in the last 3 months. Eighty per cent (20/25) had tried one or more types of antihistamine, often clemastine, hydroxyzine and levomepromazine. Of these, 9/20 (45%) had a moderate or good effect.

Compliance

All patients completed the study and none were excluded because of an acute exacerbation or an intercurrent skin infection. One patient could not attend for one of her experimental skin tests and in another case, a 7 days' Pain-Track recording was lost, because of a defective battery. In total, 443 days and nights were recorded with the Pain-Track system. The subjects' average compliance was 92·2 ± 5·4% (range 82–99%). Nine patients had one or more days with a compliance less than 70%, meaning that a total of 17/443 days (3·8%) had to be excluded. A compliance of 100% was shown on 198/443 days (44·7%). All VAS forms were completed.

Experimental flare and itch

Both terfenadine and clemastine reduced histamine-induced flare and itch significantly ($P < 0{\cdot}001$) compared with placebo (Fig. 1). Terfenadine inhibited all itch variables significantly more than did clemastine ($P < 0{\cdot}05$), but the flare reactions did not differ significantly between the drugs ($P = 0{\cdot}07$).

Clinical itch and sedation

The results of non-parametric analysis based on medians, and parametric analysis based on average values from days 2–3, were similar. No significant difference in itch intensity between the three treatment periods was detected with Pain-Track (Fig. 2a), nor was there any difference in time awake without pruritus. No significant changes in itch magnitude appeared during each period (days 0–3). Eleven of 25 patients (44%) never changed the itch intensity knob during the second or third nights of the therapies, whereas the other 14 changed it approximately every second night (range 1–5 times per night). The number of changes at night did not differ between the three treatment periods. The amount of topical hydrocortisone used during the washout after each treatment course did not differ significantly. The 3-day retrospective VAS recordings showed no difference in itch intensity between the three treatments (Fig. 2b). However, clemastine was significantly more sedative ($P < 0{\cdot}0001$) than terfenadine or placebo. The latter two did not differ significantly in this respect (Fig. 3).

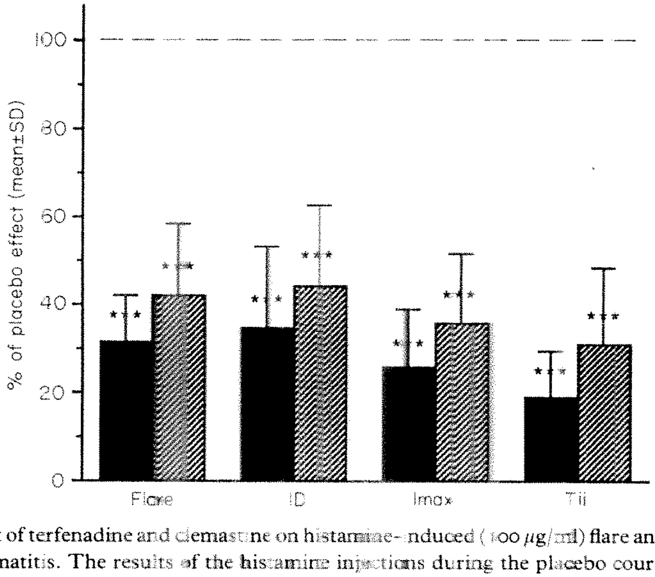

FIGURE 1. Effect of terfenadine and clemastine on histamine-induced (100 μg/ml) flare and itch in patients with atopic dermatitis. The results of the histamine injections during the placebo course are defined as 100% (– – – –). ID, itch duration (sec); Imax, maximal itch intensity (mm); Tii, total itch index (area under the curve, mm^2). *** Significantly different from placebo ($P < 0{\cdot}001$). The recordings were made on the third day of treatment, 5–7·5 h after the morning dose. ■, Terfenadine 60 mg b.i.d. for 3 days ($n = 24$); ▨, clemastine 2 mg b.i.d. for 3 days ($n = 25$).

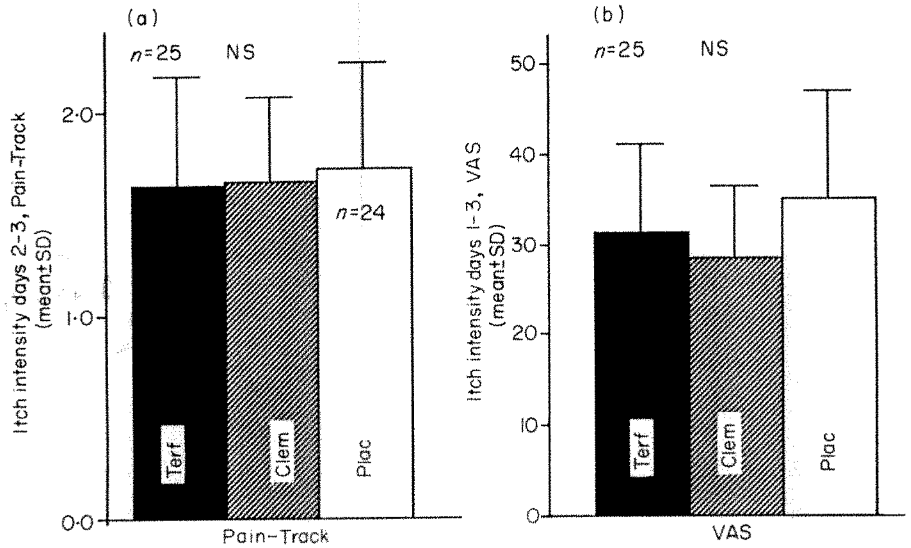

FIGURE 2. Itch intensity measured with (a) Pain-Track (samples every 10th min) and (b) VAS forms during treatment with terfenadine 60 mg, clemastine 2 mg or placebo b.i.d. in patients with atopic dermatitis. *n*, 25, except for Pain-Track, recording during placebo, where $n = 24$. NS, not significant. Terf, terfenadine; Clem, clemastine; Plac, placebo.

'Itch profile'

The 'itch profile' (Fig. 4) did not differ between terfenadine, clemastine and placebo. There was a significant increase in itch intensity during the day from 14.00 h ($P < 0{\cdot}001$). In most cases the maxima appeared within 60 min before sleep and were not inhibited by any of the antihistamines. Figure 2(a) and Figure 4 are not contradictory, as the results are based on different sampling intervals (every 10th min during time awake, and every 60th min between 08.00 and 22.00 h, respectively).

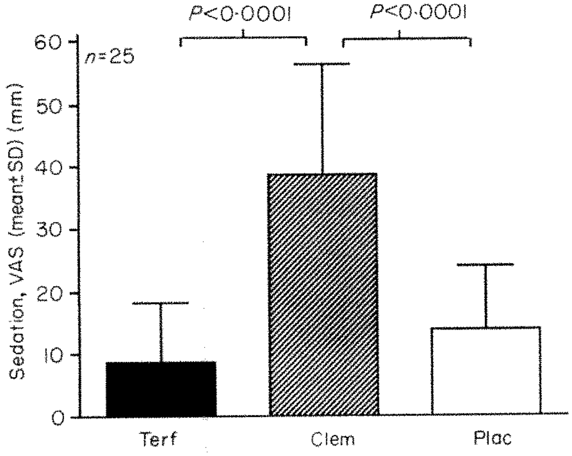

FIGURE 3. Sedation measured with VAS ratings (mm) on the third day of treatment with terfenadine 60 mg, clemastine 2 mg or placebo b.i.d.

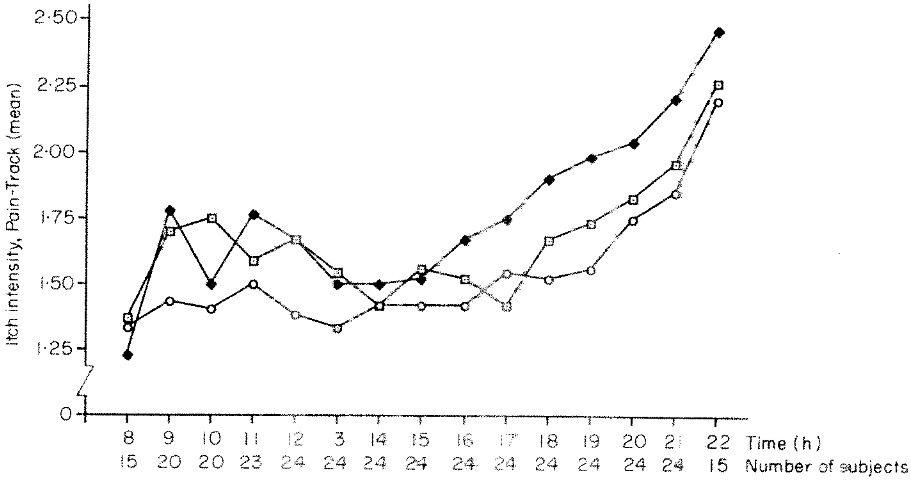

FIGURE 4. Itch profile measured with Pain-Track (samples every 60th min) on the 2 last days of treatment with terfenadine 60 mg, clemastine 2 mg or placebo b.i.d. for 3 days in patients with atopic dermatitis. ◆, terfenadine; □, clemastine; ○, placebo

DISCUSSION

In this investigation of adult AD there was no significant antipruritic effect of terfenadine or clemastine compared with placebo, whereas clemastine induced significant sedation. Intradermal skin tests showed a clear peripheral H_1 blocking effect by both antihistamines, but not by placebo. This indicates that the subjects had taken the drugs as requested, and that significant concentrations were achieved in the skin. If histamine were of importance as a major pruritic agent in AD, H_1-receptor blocking drugs would relieve itch promptly. It has been suggested that the itch-relieving effect of antihistamines could be due to a central sedative action, rather than to a peripheral H_1-receptor inhibition.[2] However, this was not confirmed in our investigation, where the sedative effect with clemastine was highly significant, but the antipruritic effect did not differ from that of placebo. Sedation at night may be beneficial for the patient by relieving anxiety and sleeplessness, without necessarily producing any relief of the itching.

The contradictory results of earlier investigations into the antipruritic effect of antihistamines in AD may be due to their different protocols, e.g. different antihistamines, doses, durations of treatment, and to the often overlooked difficulties of assessing itch. The possible existence of various subgroups of AD, with different pathophysiology, should also be taken into consideration. Doherty *et al.* recently reported antipruritic effects in AD after 10 days of treatment with acrivastine or terfenadine.[4] The results of *in vitro* experiments suggest that some antihistamines, such as terfenadine, may have mast cell stabilizing properties,[8] which prevent the release of inflammatory mediators.

The sensitivity of our methods to assess itch has been shown in a previous study of AD patients, where the capacity of Pain-Track and daily VAS forms to detect an antipruritic effect was demonstrated shortly after beginning topical treatment with betamethasone dipropionate.[7] The ability of Pain-Track and VAS to discriminate between low dosages of histamine (1–10 μg/ml) was also confirmed in a study of experimental histamine-induced itch.[6]

In conclusion, both terfenadine and clemastine had a pronounced H_1-receptor blocking effect in the skin of patients with AD, but their antipruritic effect on the patients' clinical itch did not differ from that of placebo. These findings support the view that histamine is not of great importance in the pathogenesis of pruritus in AD.

ACKNOWLEDGMENTS

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Failure of papaverine to reduce pruritus in atopic dermatitis: a double-blind, placebo-controlled cross-over study

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SUMMARY

Papaverine has been reported, largely on the basis of clinical experience, to reduce the severity of pruritus associated with atopic dermatitis. A double-blind, placebo-controlled, cross-over study was performed to assess the degree of improvement. Fifty subjects with atopic dermatitis each received papaverine 100 mg q.d.s. orally for 4 weeks and another 4 weeks of matching placebo in randomized order. The parameters used to measure response were pruritus as assessed on visual analogue scales by the subjects, clinical scoring of extent and severity of the dermatitis and rate of usage of topical steroid preparations. Forty-five subjects completed the protocol and no improvement in any parameter was demonstrated.

Atopic dermatitis is a common disease and itching is the most constant and the most distressing symptom. Any agent shown to provide even partial relief from this pruritus would be extremely valuable in the treatment of the condition. The use of papaverine was recently advocated by Baer,[1] who reported his experience in hundreds of patients over the course of 38 years. Baer reported that papaverine hydrochloride, taken by mouth in doses of 100 mg four to six times daily, was 'often effective in significantly decreasing itching in patients with atopic dermatitis'.

We performed an open study of papaverine hydrochloride 100 mg four times daily in six patients suffering from atopic dermatitis. All of these patients reported some improvement in their pruritus and in one case the response appeared dramatic. We therefore proceeded to perform a double-blind, placebo-controlled, cross-over study of papaverine in atopic dermatitis.

METHODS

Study design

In this double-blind, placebo-controlled, cross-over study the active and placebo phases were each of 4 weeks' duration.

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Subjects

Fifty subjects (29 male, 21 female; mean age 25·6 years, range 4–68 years, 12 subjects aged 12 or under) with atopic dermatitis were recruited for this study. Atopic dermatitis was defined using the criteria of Hanifin and Rajka.[2] All subjects had moderate or severe atopic dermatitis requiring treatment with moderately potent or potent topical steroids. Each subject (or a parent) gave informed consent to participation in the study. Exclusion criteria for the study were: pregnancy, an intention to become pregnant, atopic dermatitis so severe or unstable as to be likely to require hospital admission or systemic treatment likely to interfere with the study, and any coexisting disease requiring systemic therapy.

Treatment

Each adult subject received papaverine 100 mg q.d.s. orally and matching placebo, each for 4 weeks. In children aged 12 or under the dose used was 60 mg q.d.s. The order of administration was randomized by the hospital pharmacy, and equal numbers of subjects received active and placebo treatment first.

All patients were maintained on their usual treatment in addition to the trial medication. This consisted of an emollient cream, a bath oil, and a topical steroid preparation. Patients were advised to adjust the quantity of topical steroid used as necessary to control their dermatitis. No other change in treatment was allowed during the period of the study, nor any systemic treatment other than papaverine.

Compliance

Compliance was monitored by questioning patients during and after completion of each treatment phase and confirmed by tablet counts

Assessment

Each subject was provided with a diary to record the severity of pruritus during the last 7 days of each treatment period. At 08.00 and 20.00 each day subjects were asked to assess the severity of itching over the preceding 12 h by marking a point on a visual analogue scale of 10 cm in length and labelled 'no itching' at the left hand end and 'worst itching ever' at the right. Scores were calculated by measuring the distance, in cm, from the left hand end of each scale to the point marked by the patient, and summating the results to produce a maximum score of 140.

At the end of each treatment phase each subject was examined and the severity of atopic dermatitis scored. The body was assessed in 16 separate zones: each palm, the dorsum of each hand, anterior and posterior aspects of each arm, each ankle, posterior aspects of each lower limb, the abdomen, the back, the neck and the face. In each zone the extent of the disease was graded 0–3 by estimating whether none, a third, two thirds or all of the zone was affected. Additionally, in each zone the following features were assessed: erythema, excoriation, dryness, cracking, and lichenification. Each feature was scored 0 for absent, 1 for detectable, 2 for prominent and 3 for severe. A final score for each zone was obtained by multiplying the sum of the scores for disease features, by the grade for disease extent. The scores from each zone were then summated so that the maximum possible score was 720. All assessments were performed by the same clinician.

Weights of topical steroids used during the study were monitored by prescribing a recorded quantity (more than required) at the beginning of each treatment phase and weighing the unused medication at the end of each phase.

Each subject was questioned about side-effects at the end of each treatment phase. Questions were phrased in such a way as to avoid suggesting any specific symptom.

Statistical analysis

Clinical severity scores from each treatment phase were compared using the Wilcoxon signed-ranks matched-pairs test. Pruritus scores were compared in the same way, as were the total amounts of topical steroid applied during each phase of the study. This non-parametric test was used as described by Koch,[3] since there was no guarantee that the data would be normally distributed. Clinical scores, pruritus scores and steroid usage were each analysed for evidence of a carry-over or period effect by the method of Hills and Armitage.[4]

RESULTS

Forty-five of the 50 patients completed the study having complied fully with the protocol. None of these 45 patients had omitted to take more than four capsules (400 mg, or one day's medication) over the course of any one treatment phase. Five subjects were eliminated from the analysis due to failure to comply with the protocol, either by failing to take the trial medication as directed, by failing to attend for review at the correct time or by changing other treatment during the study.

The mean pruritus scores, and standard errors of the means, are shown in Table 1. Twenty subjects recorded higher pruritus scores on placebo than papaverine, and 25 recorded higher scores on papaverine. There was no significant difference between scores on papaverine and placebo.

The mean clinical scores, and standard errors of the means, are given in Table 1. Twenty-one patients were clinically better after the active phase and twenty-four were better after the placebo phase. There was no significant difference between the active treatment and placebo phases.

Three subjects failed to return their topical steroid medication for weighing as directed. Of the remaining 42 subjects, 26 used 0·1% diflucortalone valerate (Nerisone Oily Cream), 10 used preparations classified as 'potent' and therefore in the same group as diflucortalone valerate,[5] and six subjects used 1% hydrocortisone butyrate (Locoid Lipocream), a 'moderately potent' preparation.[5] Twenty-one patients used more topical steroid during the active phase and 21 used more during the placebo phase. No significant difference in the rates of usage of topical

TABLE 1. Summary of results

	Active phase (mean ± SEM)	Placebo phase (mean ± SEM)
Pruritus score (Max = 140)	58 6 (±4 73)	55 7 (±4·43)
Clinical score (Max = 720)	178 (±14 1)	176 (±16·1)
Topical steroid usage (g per month)		
Potent group (*n* = 36)	60 (±9)	58 (±8 6)
Moderately potent group (*n* = 6)	46 (±27·8)	30 (±13·4)
Total (*n* = 42)	58 (±8 6)	55 (±7·7)

steroids could be demonstrated between the active and placebo phases when the rates for the potent and moderately potent groups were compared either separately or together. There was no significant carry-over or order effect in any of the parameters.

The power of the study to detect a 25% improvement in the pruritus score was estimated using the mean score on placebo and the standard error of this mean by the method described by Hills and Armitage.[4] With 45 subjects completing the study the power was over 85%. The power of the study to detect a 25% improvement in the clinical score, assessed by the same method, was between 75 and 80%, and to detect a 25% reduction in total steroid usage it was between 35 and 40%.

Side-effects

The side-effects reported while taking papaverine were (each in one subject): a 'mild calming effect' which the subject considered beneficial, unusual thirst, poor concentration, slight drowsiness, mild abdominal pain and nausea. Side-effects reported while on placebo were (in one case each): slight tiredness, slight insomnia, severe insomnia (this subject was withdrawn from the study by his general practitioner), indigestion and heartburn, nausea with vomiting and abdominal pain lasting 1 week.

DISCUSSION

Papaverine is a naturally occurring compound found in opium but lacking any narcotic activity, and appears to be a remarkably safe drug.[1] It is a potent inhibitor of phosphodiesterase and it is this property that provides a possible mechanism of action in atopic dermatitis, as elevated phosphodiesterase in mononuclear leucocytes and basophils may be significant in the pathogenesis of the disease.[6]

The use of papaverine for relief of pruritus was first described by Wirth[7] in 1947. This author investigated a number of agents believed to relieve pruritus through a vasodilating action and concluded that the safest of these was papaverine. He administered papaverine either orally or intravenously to patients complaining of pruritus due to infectious hepatitis and a variety of dermatoses. Pruritus was improved in each of 29 cases and the response was 'marked to complete' in 26 of these. However, this study was not controlled in any way. The use of papaverine in pruritus was further investigated by Baer,[1] who found it to be effective only in atopic dermatitis, but this conclusion appears to have been based on clinical experience, rather than on controlled studies.

In this study papaverine was used in the dose range and frequency as stated by Baer.[1] The results provide no support for the use of papaverine to relieve pruritus or to produce any other benefit in atopic dermatitis. Whilst we accept that there are always inherent difficulties in the measurement of pruritus and the clinical scoring of the severity of dermatitis, and that the available methods have limited sensitivity, the results of this study were conclusively negative.

Our own initial success using papaverine in an open manner was probably a placebo response and demonstrates how essential it is to perform a controlled study when evaluating a treatment for atopic dermatitis.

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Xeroderma pigmentosum variant: response to etretinate

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SUMMARY

We report a case of the variant form of xeroderma pigmentosum in whom tumour development was completely suppressed for 22 months by etretinate 25 mg daily.

The clinical phenotype known as xeroderma pigmentosum represents a rare group of inborn errors of DNA repair. These result in a markedly increased tendency to develop cutaneous malignancies following exposure to ultraviolet light. An extremely rare form of xeroderma pigmentosum results from a defective mechanism for joining up daughter strands of DNA synthesized to repair damage induced by ultraviolet light.[1] This variant typically has a later onset than other forms and tends to be less severe.[2] Nonetheless, as our case demonstrates, this variant may result in life-threatening malignancy, and an effective prophylactic treatment for neoplasia is of immense value. We believe this is the first report of the variant form of xeroderma pigmentosum responding to etretinate.

CASE REPORT

Mr A.M., now aged 63, developed a cutaneous neoplasm at the age of 21, after completion of a 5-month period of military service in Palestine. This lesion was on the side of the nose, and was treated by curettage. It recurred very rapidly, growing to 3 cm in diameter within 2 weeks, then spontaneously resolving. No firm diagnosis was made. Subsequently, and with increasing frequency, he developed many further cutaneous neoplasms on his face and the dorsal aspects of his hands and forearms. Tumours histologically documented at the Leicester Royal Infirmary have included 13 keratoacanthomas, three actinic keratoses, two basal cell carcinomas, eight squamous cell carcinomas and recently, a superficial spreading malignant melanoma. Numerous other lesions have been treated at other hospitals.

In his youth he readily developed a suntan after 4–6 h of exposure to minimal sunshine and was not aware of any undue sensitivity to sunlight. There was no family history of skin tumours. Over the years, the skin of his face and hands had become markedly freckled and telangiectatic,

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TABLE 1. Mean molecular weights of DNA daughter strands (in megadaltons) following UV irradiation

	Standard medium	Caffeine added
Patient	131	51
Normal control	153	147
XP variant control	120	57

and he had the typical cutaneous features of xeroderma pigmentosum. In 1969 he was found to be mildly sensitive to UVB (300 nm), but had normal reactions to wavelengths of 260 nm and 320–700 nm.

The synthesis of DNA induced in fibroblasts by germicidal UV irradiation was normal. An assessment of post-replication DNA repair revealed low molecular weight daughter strands compared to a normal control, but similar to a control with variant xeroderma pigmentosum (Table 1). This delay in joining daughter strands was enhanced by caffeine.

In spite of exceptional compliance with advice to avoid sunlight and to constantly use sunscreens, he has required at least 90 surgical procedures for skin neoplasms, received radiotherapy on six occasions and innumerable treatments with cryotherapy and topical 5-fluorouracil. In October 1987, following excision of a malignant melanoma from the forearm, treatment was started with etretinate 25 mg daily. This treatment has been well tolerated and has not been interrupted. No further neoplasms have developed in the past 22 months.

DISCUSSION

This patient had a history typical of the variant form of xeroderma pigmentosum. His dermal fibroblasts showed normal DNA synthesis following irradiation, excluding the classical forms of this disease. However, fibroblast cultures did reveal the delay in joining daughter strands of DNA, enhanced by caffeine, which is characteristic of the variant form of the disease.[1] He fulfilled all the diagnostic criteria for the variant form of xeroderma pigmentosum.

In the 12 months prior to starting treatment with etretinate our patient developed nine separate skin tumours requiring treatment, and the frequency of these neoplasms was increasing. After starting etretinate no new lesions have developed in the past 22 months and this represents an unequivocal response to treatment.

Etretinate has been reported to show an anti-neoplastic effect in a variety of premalignant skin diseases including the classical forms of xeroderma pigmentosum.[3-5] In most of these reports, doses of 0·75–1·0 mg/kg per day were required. The comparatively small dose which has proved effective in our patient (0·33 mg/kg per day), suggests that the variant form is particularly responsive to this treatment.

ACKNOWLEDGMENTS

We thank Dr F.B.Giannelli of Guy's Hospital, London, for performing the assay of post irradiation DNA synthesis on our patient, and Drs C.F.Arlett and A.R.Lehmann, of the University of Sussex, for performing the assay of post-replication repair.

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Congenital self-healing Langerhans cell histiocytosis with persistent cellular immunological abnormalities

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SUMMARY

We describe an infant who presented at birth with numerous haemorrhagic and encrusted skin lesions, hepatomegaly, lymphadenopathy, raised hepatic transaminases, leucopenia and thrombocytopenia. The diagnosis of Langerhans cell histiocytosis was confirmed by immunohistochemistry, which demonstrated the presence of CD1, S-100 and DR positive cells in the skin infiltrate. The skin lesions resolved spontaneously after 6 weeks but recurred at 3 months and again were self involuting with resolution by 9 months. Persistent circulating T-cell abnormalities, including T-cell lymphopenia and the presence and persistence of peripheral blood CD1$^+$ cells were noted throughout the first year of life.

The term 'Histiocytosis X' has been replaced by 'Langerhans cell histiocytosis'.[1] The change in classification is an indication of the diversity of this group of diseases. The pathology seems to be similar, but clinical expression is governed by the type as well as the number of organs affected and the degree of the histiocytic infiltration.

Congenital forms of the disease are rare, involve mainly the skin and are usually rapidly healing and non-progressive. They were originally described as congenital self-healing reticulohistiocytosis in 1973.[2] It would now seem more appropriate to refer to this disease as congenital self-healing Langerhans cell histiocytosis. Immunological abnormalities persisting after resolution of all skin lesions have not previously been described.

CASE REPORT

A female infant was born at term to a gravida 3, para 2, 32-year-old at Queen Charlotte's Maternity Hospital, London. Amniocentesis was performed at 16 weeks, as there had

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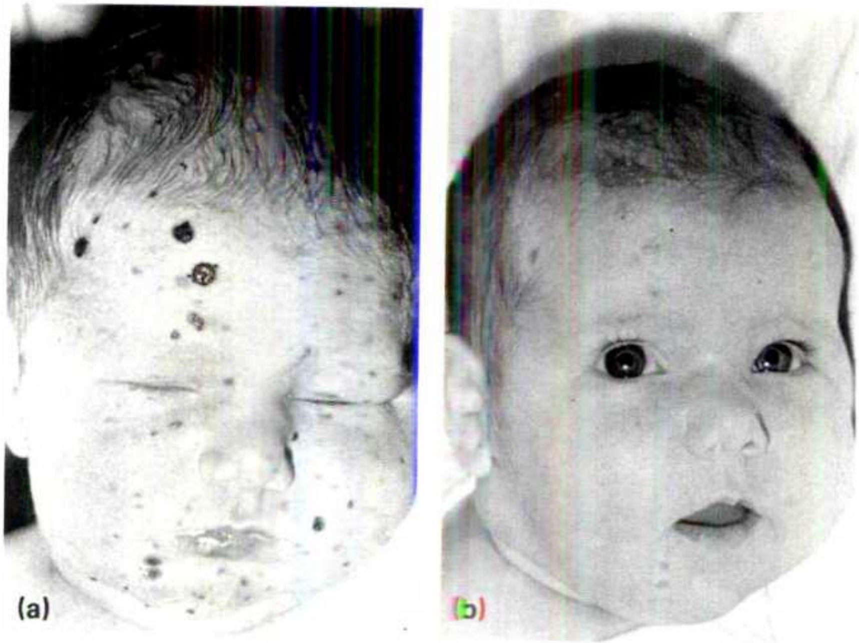

FIGURE 1. (a) Cutaneous lesions over face present at birth. (b) By six weeks of age the lesions had almost completely resolved.

previously been a Down's syndrome child, and it revealed a normal female karyotype. The mother was rubella immune, VDRL negative and had no other complications during the pregnancy. The baby had Apgar scores of 8 at 1 min and 9 at 5 min. Her weight was on the 90th centile, height on the 75th centile and occipito-frontal circumference on the 97th centile. She was active and apyrexial.

A widespread skin rash was noted at birth. This consisted of numerous papular, haemorrhagic and encrusted lesions measuring 0·5–1 cm in diameter and involving the entire body, though more prominent at the extremities (Fig. 1a). Associated with this rash was hepatosplenomegaly, the liver being 6 cm and the spleen 1 cm below the costal margin. Marked lymphadenopathy was demonstrated in both the axillary and inguinal regions. Examination of the eyes revealed no evidence of cataracts or retinopathy. There were no other abnormalities noted.

Histopathology of two papular lesions from the lower limbs showed an intact epidermis with a mixed inflammatory infiltrate and collections of histiocytes and giant cells in the dermis and subcutaneous tissue. The histiocytic cells had abundant cytoplasm and prominent, convoluted nuclei (Fig. 2). Immunostaining for S-100 protein and CD1 (T6) antigen showed that the majority of these cells were positive. In serial sections, approximately one-third of the cells were also DR positive. However, on electron-microscopy no Birbeck granules were seen in these cells though they were present in the normal Langerhans cells of the epidermis. This observation was confirmed by examination of serial ultra-thin sections. The absence of Birbeck granules is in contrast to other descriptions of congenital self-healing reticulohistiocytosis where they have been noted to be present.[2,3]

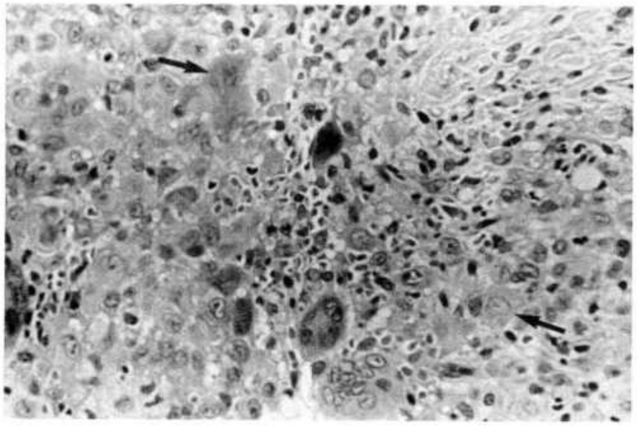

FIGURE 2. Section of skin obtained from a papular lesion of leg (H&E). Note the large histiocytes with prominent cytoplasm (arrowed).

Initial full blood count in the first week of life showed haemoglobin 13·5 g/l, white cell count $2{\cdot}4 \times 10^9$/l, neutrophils 56%, lymphocytes 31%, monocytes 10% and eosinophils 1%. Platelets were 84×10^9/l. Despite the leucopenia and thrombocytopenia, bone marrow examination showed normal cellularity. The slight enlargement of the spleen was insufficient to account for these findings. The hepatic transaminases were elevated with an ALT of 119 U/l and an AST also of 119 U/l. The serum albumin was 27 g/l and the urea, electrolytes, creatinine and clotting screen were all within the normal range. Antibody titres against toxoplasma, rubella, cytomegalovirus and herpes simplex virus reflected maternal transfer with no evidence of foetal infection. Viral cultures were also negative. Bacteriological swabs of the lesions grew *Staphylococcus epidermidis* only. Chest X-ray and skeletal survey showed no evidence of infiltration or abnormal calcification, although the bone age was delayed at 30 weeks. A CT scan of the head revealed no abnormalities. Ultrasound of the abdomen demonstrated hepatomegaly but showed a normal echogenic pattern.

The infant continued to thrive and the skin lesions spontaneously involuted showing almost complete resolution by 6 weeks of age (Fig. 1b). The lymphadenopathy and hepatosplenomegaly also resolved. From the age of 3 months however, new lesions began to reappear on her face, scalp and ears. These were not as pronounced as at first presentation, the papules being smaller and not haemorrhagic in appearance. They too spontaneously resolved by 9 months and have not recurred. There was no associated systemic involvement and she has continued to thrive without treatment. Clinical follow up at 1 year of age revealed a well child.

Immunology

The initial low total white cell count ($2{\cdot}4 \times 10^9$/l) was contributed to mainly by a lymphopenia ($0{\cdot}7 \times 10^9$/l). This was present when the skin symptoms were most florid. Total white cell count and lymphocyte count returned to normal levels by the age of 3 months (Fig. 3). Despite a normal total lymphocyte count, there was a low percentage of $CD3^+$ cells. The absolute numbers of CD3 cells did increase with time but have remained below the normal range for age-matched children (Fig. 4). The CD4:CD8 ratio was within the normal range, although the absolute numbers of these subsets were below normal. The combined numbers of $CD4^+$ and

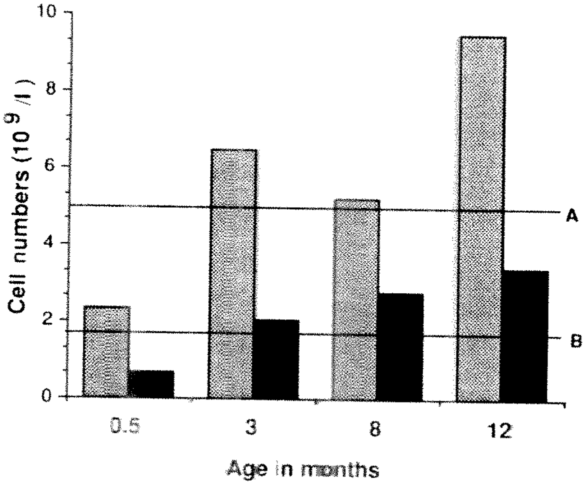

FIGURE 3. Total white cell count and total lymphocyte count ($\times 10^9$/l) of patient over first year of life. Lines A and B represent lower normal of total white cell count (5×10^9/l) and total lymphocyte count ($1{\cdot}5 \times 10^9$/l) respectively. ▨, total white cell count; ■, lymphocytes.

CD8$^+$ cells exceeded the total number of CD3$^+$ cells. This seemed most likely due to CD16 and CD8 double-staining cells, which would suggest an expanded large granular cell lymphocyte population with natural killer activity. However, double-staining procedures and functional analyses have not been performed. Although CD1$^+$ cells have been described in cord blood,[4,5] routine fluorescence-activated cell-sorting analysis of blood from age-matched infants on the

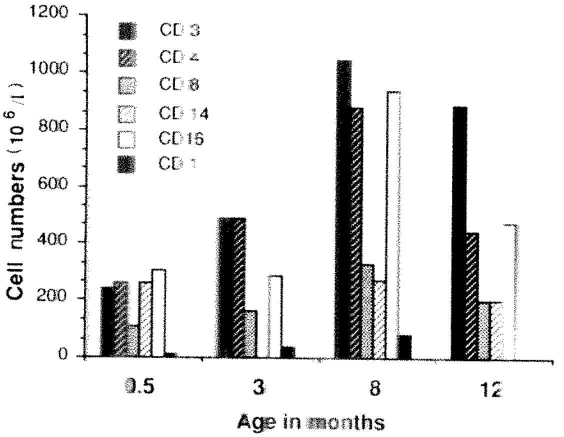

FIGURE 4. Absolute number of circulating T-cell subsets over the patient's first year of life. Normal ranges of absolute numbers for age matched children: CD3, 3960–5180 $\times 10^6$/l; CD4, 2640–4230 $\times 10^6$/l; CD8, 780–1750 $\times 10^6$/l; CD14, 120–730 $\times 10^6$/l; CD16, 720–1680 $\times 10^6$/l. CD1 cells are not normally detected in the peripheral blood of infants.

same day consistently failed to demonstrate circulating CD1$^+$ cells. This is suggestive of an increased level of circulating CD1$^+$ cells whose origin and function (immature T cells, Langerhans cells or a B-cell subset) remain unclear.

The number of cells which expressed CD14 (mainly expressed on monocytes and epidermal Langerhans cells) remained within the normal range. Cells expressing CD16 (FcR III receptor) on natural killer cells, granulocytes and macrophages were initially low, though reached normal levels in the latter part of the first year (Fig. 4).

The reduced number of T-lymphocytes was associated only with a marginal reduction of the PHA responsiveness during the first 3 months of follow-up. Complement studies and immunoglobulins were normal. The nitro-blue tetrazolium test and opsonization measured by C3b uptake were also normal throughout the first year.

Histiocytic cells were extracted from skin biopsy specimens by panning. Eighty per cent were CD1(T6)$^+$. Functional studies performed on these, including alloantigen stimulation and mitogenic responses, showed good accessory function for antigen presentation. This was in contrast to other patients with later onset, generalized Langerhans cell histiocytosis (of the Letterer–Siwe type), whose cells are non-functional (Dr A.Chu, personal communication).

DISCUSSION

The original case report[2] of congenital self-healing reticulohistiocytosis had a presentation at birth with skin lesions consisting of multiple dark red papulo-nodules with or without ulceration. There was no systemic involvement and the lesions healed spontaneously within 2–3 months with no recurrence. This is a unique form of Langerhans cell histiocytosis which is benign, probably representing a self-rectifying congenital anomaly of the histiocytic system.[6] Since then, there have been other cases reported in the literature.[3,7–9] Even though this is a rare condition, it is of interest as it spontaneously resolves and there is the potential for overtreatment.

Hepatomegaly and haematological abnormalities have been previously reported in association with congenital self-healing reticulohistiocytosis.[3,7] Altered CD4:CD8 ratios have also been demonstrated,[10] although decreased absolute CD3 cell numbers and the persistence of CD1 (T6 positive cells) have not previously been shown. These findings in our patient represent immunological abnormalities which after 12 months of follow-up had not completely resolved despite the child thriving and being symptom-free. It is tempting to speculate that the persistence of the increased numbers of CD1$^+$ cells in the blood may be correlated with the skin changes (CD1$^+$ cell infiltration).

The case discussed here is also unusual in that the skin lesions recurred at 3 months of age. There have been several previous reports of a benign cutaneous variant of Langerhans cell histiocytosis with an onset later in infancy[11,12] and in two cases the recurrence of skin lesions after a disease-free period of 22 and 24 months respectively.[12]

The 'self-healing' nature of congenital Langerhans cells histiocytosis is fascinating as not only do the cutaneous forms of this disorder resolve spontaneously but this may occur with multisystem disease.[13] At present the prognosis and treatment regimen for Langerhans cell histiocytosis are decided by clinical presentation as well as the histological appearance.[14,15] We feel that further immunological investigation will lead to a better understanding of the aetiology of this disease spectrum.

ACKNOWLEDGMENTS

We wish to thank Dr D.Harvey (Queen Charlotte's Hospital) for referring this patient; Professor B.Lake (histochemistry), Professor A.Risdon and Dr M.Malone (histopathology), Department of Medical Illustration, Hospitals for Sick Children; Dr A.Chu (Hammersmith Hospital) for the Langerhans cell function studies; and Miss Paula Smith and Miss Leigh Stanger for secretarial assistance.

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PEDIATRIC DERMATOLOGY

Editors

Lawrence M. Solomon *Department of Dermatology,*
University of Illinois Medical Center
Nancy B. Esterly *Department of Pediatrics, Children's Hospital of Wisconsin*

Pediatric Dermatology is an international journal which is closely associated with the Society for Pediatric Dermatology and the International Society of Pediatric Dermatology. Its primary purpose is to publish original papers relating to clinical and laboratory investigations, case reports, review articles relevant to the speciality of paediatric dermatology, editorial comments and book reviews.

Pediatric Dermatology aims to further the knowledge of both paediatricians and dermatologists regarding skin diseases of infants, children and adolescents. It particularly supports the contention that skin diseases are ultimately related to the workings of the organism as a whole and may reflect physiological as well as psychological alterations. The journal therefore welcomes contributions which are critical, authoritative and directly related to the structure, function and diseases of the skin. These studies may be biochemical, pharmacological, histological, immunological, electron microscopic, or clinical in nature.

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British Journal of Dermatology (1990) **122**, 569–572.

Correspondence

Psoriasis, nails, joints and autoimmunity

SIR, Fifty-four consecutive in- and out-patients were examined over a 3-month period for evidence of nail changes and interviewed concerning arthropathy. Twenty-four of these patients (13 male and 11 female; age range 16–82 years, mean 51 years) had the triad of psoriasis, nail changes and arthropathy and they were further studied for familial evidence of autoimmunity. Five patients were suberythrodermic, seven had severe, widespread plaque-type psoriasis and 12 moderate but widespread plaque-type psoriasis. Blood was taken for HLA studies. It was found that 14 patients had a family history of autoimmune diseases (five thyroid disease, four diabetes, three rheumatoid arthritis, and two with pernicious anaemia) compared with a similar age–sex-matched control group without psoriasis and who had a family history of autoimmune diseases in only five out of 24 patients ($P < 0{\cdot}0001$). Eight of the study group of patients had a strong family history of psoriasis, nine were female (seven aged less than 50), 10 had mild to moderate nail and joint changes, and 10 had large joint arthropathy with or without small joint changes.

There is evidence that an autoimmune process may be important in the pathogenesis of psoriasis. Severe psoriasis can be associated with arthritis, a condition which is commonly assumed to be immunologically mediated.[1] It is also established that psoriasis, like a number of other possible autoimmune conditions, is associated with certain HLA phenotypes[2–4] and studies have shown an increased prevalence of HLA B_{17}, HLA B_{13}, HLA B_{27} and HLA CW_6. In the present study, there was a strong statistical association of psoriasis with HLA B_{17} ($P < 0{\cdot}001$) and weaker associations with HLA A_{29} ($P < 0{\cdot}005$), HLA B_{27} ($P < 0{\cdot}005$) and HLA B_{37} ($P < 0{\cdot}05$). Recent work has shown that early plaques of psoriasis are infiltrated by T-helper (T_h) lymphocytes, while T-suppressor (T_s) lymphocytes predominate in regressing lesions.[5,6] There are increased numbers of HLA DR^+ dendritic cells in psoriatic plaques and cyclosporin, a drug which acts selectively on T_h lymphocytes, is useful in treating severe psoriasis.[7] It has been suggested that the interaction of T_h lymphocytes with antigen-presenting dendritic cells could lead to the release of lymphokines and induce keratinocyte proliferation.[6] The age incidence of psoriasis is consistent with the hypothesis of autoimmune pathogenesis,[8,9] the incidence rising with age to a peak in the second or third decades and thereafter becoming less common.

In this limited study we found that some patients with severe psoriasis have an increased incidence of other autoimmune diseases in the close relatives. Young females with widespread plaque-type or suberythrodermic psoriasis and with a strong family history of psoriasis and who have both nail changes and large joint arthropathy may form a separate subgroup of patients with a strong family history of autoimmune disease.

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Satisfactory resolution of Jessner's lymphocytic infiltration of the skin following treatment with etretinate

SIR, We wish to report a case of Jessner's lymphocytic infiltrate (JLI) which responded well to etretinate. The condition is a benign disorder characterized by areas of raised discoid erythema on the face and trunk,[1] and often occurs in the light-exposed areas of the skin. The histology typically shows a dermal perivascular lymphoid infiltrate consisting mainly of T cells.[2] The condition tends to run a relapsing course and is often difficult to treat.

Our patient was a 35-year-old male who had raised areas of erythema on the left cheek and temple, which had been present for 1 year and which were conspicuous. An initial biopsy showed appearances compatible with JLI and a second biopsy several months later confirmed the clinical diagnosis. Treatment with topical and intradermal steroids gave some temporary improvement, but the condition repeatedly relapsed and it was then decided to use etretinate 50 mg daily. After 3 months, the dose was reduced to 25 mg daily and continued for a further 3 months. There were no significant side-effects. The JLI subsided within 4 weeks of starting etretinate and resolved completely during the following 5 months, by which time the skin of the left cheek and temple appeared normal. Treatment with etretinate was stopped and 9 months later there has been no recurrence.

Retinoids have been used in many different skin disorders and reports of their use in discoid lupus erythematosus (DLE) have indicated varying degrees of success. Etretinate was found to be useful in resistant forms of chronic DLE, although hyperkeratotic forms were more responsive than inflammatory forms.[3] A study of 19 patients with different variants of cutaneous LE who were treated with etretinate showed good results in all patients within 2–6 weeks.[4] The best responses were seen in males with DLE. In view of the similarities between DLE and JLI, etretinate was a reasonable choice of treatment in our patient when topical steroids had failed. A very satisfactory response was achieved and there has been no recurrence to date.

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Diffuse cutaneous mastocytosis, 22 years on

SIR, A young male was presented to the Section of Dermatology at the Royal Society of Medicine in September 1968 and the case was subsequently published.[1] He was then 9 months old and the main problem was itching with wealing and blisters and with linear excoriations. Minor trauma was followed by weals and bullae. His skin showed a tanned 'peau d'orange' appearance. There was no hepatosplenomegaly. Investigations at that time were as follows: Hb 80%, WBC 23 000 mm^3 (P 17%, L 76%, M 6%, E 1%). There was no increase in the number of basophils and the lymphocytes showed a normal morphology. X-rays showed no bone or joint changes. A skin biopsy showed there was a diffuse sheet of cells with deeply staining nuclei immediately below the epidermis filling the upper half of the dermis and also in a perivascular distribution. The cells were round or spindle shaped and Giemsa staining showed the presence of metachromatic granules indicating that they were mast cells (Fig. 1).

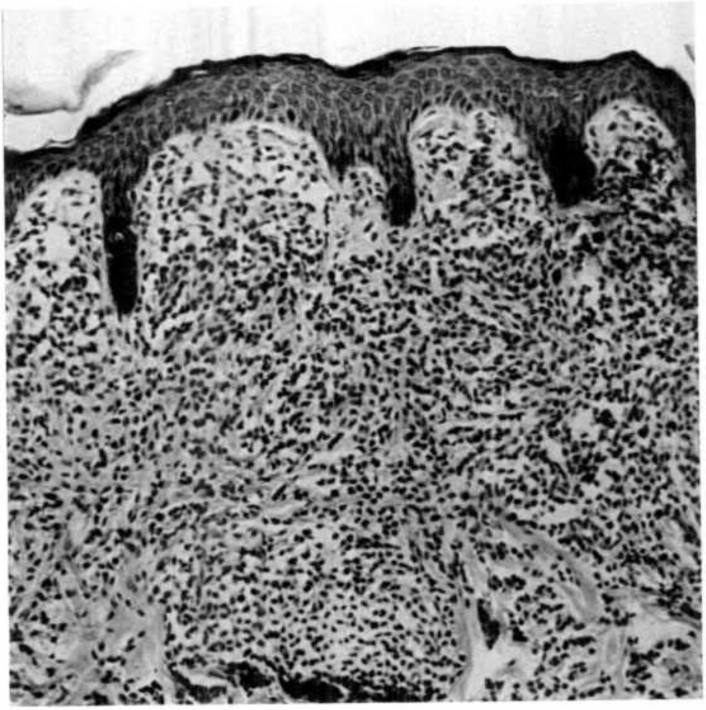

FIGURE 1. Diffuse dermal mast-cell infiltrate at 9 months of age.

His skin condition slowly improved and as he has grown up he has learned to live with the dermographism that is now not severe. He presented to us in 1988 with psoriasis. A skin biopsy was taken from a non-psoriatic area with the purpose of comparing the present histology with that seen in 1968. A moderate number of mast cells were seen in the dermis mainly around small blood vessels (Fig. 2).

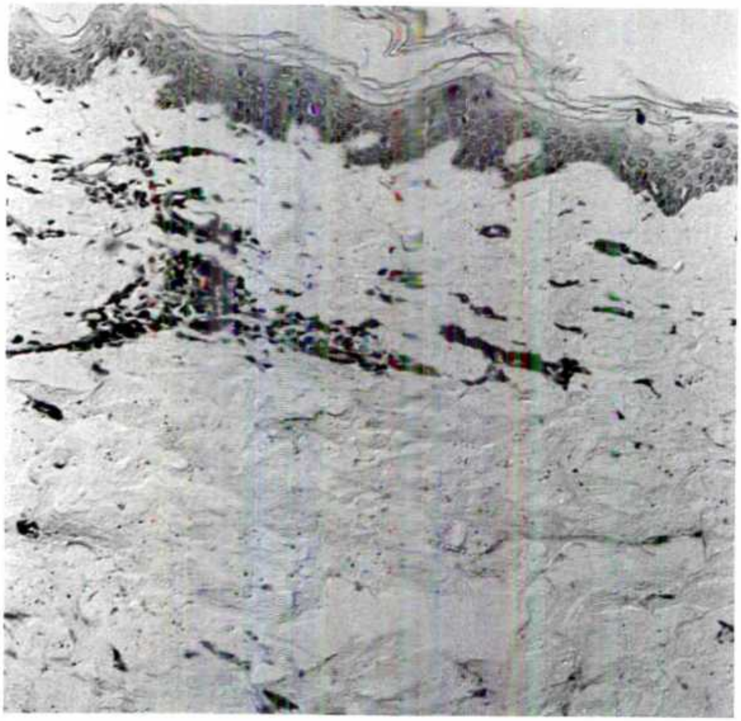

FIGURE 2. Residual perivascular mast cells at 22 years of age.

We report the change in the clinical and histological appearance of the skin of a patient who had severe diffuse mastocytosis shortly after birth and who, over the years, has improved.

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1 Beer WE, Emslie ES. Diffuse cutaneous mastocytosis. *Br J Dermatol* 1968; **80:** 841–3.

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Book Reviews

Differential Diagnosis in Dermatopathology II. A.B.ACKERMAN, J.L.TROY, L.B.ROSEN, S.JERASUTUS, C.R.WHITE AND D.F.KING (1988). Philadelphia: Lea and Febiger. Pp. 202. Illustrations 360.

This second volume follows the same format as Volume I published in 1982. In each of 45 chapters the histological features of two diseases are compared using colour photomicrographs and lists of major differentiating criteria. Each chapter is supplemented by a helpful discussion on the two diseases in question.

The quality of the photomicrographs and their uniformity of colour is excellent. Some of the differential diagnoses discussed are of diseases which can be notoriously difficult to separate. Into this category fall the chapters on psoriasis vulgaris vs. pityriasis rubra pilaris, erythema mutiforme vs. fixed drug eruption and scleroderma vs. sclerodema. These chapters are likely to be of practical use and interest to all dermatopathologists. I am less certain, however, that in practice there are many problems in differentiating granuloma annulare from eruptive xanthoma or warty dyskeratoma from acantholytic squamous cell carcinoma.

In the introduction Dr Ackerman has defended the now traditional absence of reference lists in his books by declaring that the data presented are his own. He does not refrain, however, from making dogmatic and sometimes unsubstantiated statements, for instance that tricholemmomas and inverted follicular keratoses are all old viral warts, without any indication that these are controversial views which are disputed by many.

The difficulty with this book is in determining who is likely to derive most benefit from it. I do not consider it to be a beginner's text but experienced dermatopathologists are likely to find it of limited use as a 'bench book'. I know from experience that many trainee pathologists and dermatologists with a working knowledge of histopathology find Dr Ackerman's style of concise lists of major histological features each clearly demonstrated in photomicrographs to be extremely helpful and for this group the book would make an interesting addition to departmental libraries.

MORAG SEYWRIGHT

Cutaneous Electrosurgery. J.E.SEBBENS (1989). Chicago–London: Year Book Medical Publishers Inc./Wolfe. Pp. 211. Illustrations: 111. ISBN 0-8151-7804-2. Price £39.50 or £42.00.

Do you know the difference between electrocautery, electrofulguration, electrocoagulation, electrodesiccation, electrocutting, galvanic surgery, electrolysis, and the hyfrecator? If you want to know the answer in detail, then look no further, for it is all here, down to fairly minute detail. For example, electrofulguration comes from the Latin root (fulgur—lightning) and indicates that a spark jumps between electrode and tissue. This tends to produce more damage than electrodesiccation, (where the electrode actually touches the tissue), or electrocoagulation (usually performed at a lower voltage than electrodesiccation and electrofulguration).

Or, again, did you know that galvanism is basically the same as iontophoresis? I didn't until I read this book, and I have learnt a great deal about the theoretical and physical background to electrosurgery.

The safety aspects are well covered, right down to the risk of igniting bowel gas. The practice exercises are good, and should perhaps be considered for the British Dermatological Surgery Group workshop in the future. A minor criticism is that electrocautery, which is simple, cheap, and still popular in the U.K., is hardly mentioned, and dismissed as 'not widely used today'. The section on treatment of different types of lesions is somewhat repetitive.

This is a book for the real enthusiast, but most dermatologists will find all, and more than they need to know, in a textbook such as Richard Bennett's '*Fundamentals of Cutaneous Surgery*' (1988, The C.V.Mosby Company) at approx. £70.00, which covers the subject very satisfactorily, and has the added advantage of covering the whole of dermatological surgery, which, in my opinion, makes it a better bargain.

P.W.BOWERS

Textbook of Pediatric Dermatology. Edited by R.Ruiz-Maldonado, L.C.Parish and J.M.Beare (1989). Philadelphia: Grune & Stratton. Pp. 863. ISBN 0-8089-1863-X. Price £114.00.

In the past 2 years two major paediatric dermatology texts have been published and the enthusiast now has a choice. The text reviewed here is edited by three distinguished individuals whose theme appears to be dermatology for paediatricians. This would explain the standard dermatology textbook introduction on skin biology, terminology, histology and basic treatment. The bulk of the book is divided into 18 sections devoted mainly to disease categories by aetiology, although there is a section on general dermatology. Some readers may query the inclusion of acne in the section on Diseases of Skin Structure—Non-hereditary.

There are good sections on connective tissue diseases, psoriasis and deep fungal infections. The section on atopic dermatitis was a little disappointing in relation to the frequency with which both paediatricians and paediatric dermatologists have to deal with the problem. PUVA is mentioned as a method of managing atopy and so also are cyclophosphamide and azathioprine, with the rider that they should not be used in children!

The multi-author nature of the book has led to repetition in several chapters, which could have been corrected by some stringent editing. The pictures are all black and white and many are of rather poor quality. This is particularly unfortunate as many illustrate relative rarities. The rapid development of high quality and relatively inexpensive colour printing techniques should stimulate authors, editors and publishers to consider very carefully whether or not a smaller number of high quality colour pictures would have been more valuable at the same cost.

While this book has some useful sections, the definitive paediatric dermatology textbook has yet to be written. Those who plan to write such a text should consider whether they are writing dermatology for paediatricians or paediatrics for dermatologists as the approaches will be different. Those with tight departmental or personal library budgets should examine the book and consider whether or not its contents will add significantly to what is found in the major general dermatology texts.

Rona M.MacKie

Formaldehyde Sensitivity and Toxicity. Edited by Susan. E. Feinman (1988). Florida: CRC Press, Inc. Pp. 221. ISBN 0-8493-4789-0.

This is an energetic summary of formaldehyde and its related compounds as is known today. The editor and her fellow contributors, who are also non-medical, have collected very carefully and precisely all the relevant data and arranged these in clear easily identified categories. Despite the fact that the contributors are non-medical, they are highly sympathetic to the biological effect of formaldehyde and so every aspect of this compound's effect on biological systems has been covered.

Sections headed Allergenicity, Toxicology, Allergy, Contact Dermatitis and Other Health Effects, are covered. The majority of the book deals with the dermatological aspect of contact with formaldehyde and its related compounds, therefore covering irritant and allergic contact dermatitis, both occupational and non-occupational. As well as giving the physiological and toxicological background to this, it is an extremely useful summary of the sources of formaldehyde in our average environment and in our occupational environments. For this reason the book is essential for anyone working in environmental dermatology and occupational dermatology in particular.

The authors and contributors have indicated that they would aim their text at many different disciplines, including medical, biological, chemical, legal and undergraduate students from all categories. The way the material is presented would make all of these groups aware of their own particular aspects of the compounds behaviour, and therefore is successful in that aim.

The book forms part of a series, most of which gives sound references, so it is unfortunate that Dr Etain Cronin's name is misspelt when her full name is used in an acknowledgement after Chapter 6, but this is not in keeping with the general accuracy of the volume. The book has been very careful in its representation of the current state of knowledge world-wide on formaldehyde, not only in the study of the effects of the formaldehyde on the biological systems, but possible ways of its removal.

Angela Forsyth

Surgical Gems in Dermatology. Edited by PERRY ROBINS (1988). New York: Journal Publishing Group. Pp. 118. Numerous illustrations. ISBN 0-9621300-0-1.

There are many practical tips, anecdotes and experiences that are exchanged informally between colleagues that would never usually get into a refereed journal or a standard textbook. Dr Perry Robins has gathered several of these together in this slim volume. He promises this to be the first in a series of such books. There are a total of 40 contributors to this book. The subjects range from tips applicable to all, such as reducing the pain of injecting local anaesthetic, to the esoteric, such as advice on how to drill the frontal bone of the skull to create a sling suture.

This book does reflect the difference in practice of dermatological surgery between the U.K. and North America. The book is aimed at the North American dermatological surgery trainee and office practitioner. Many of the 'Gems' apply to techniques that would not be considered the remit of a dermatologist practicing surgery in the U.K.

Each 'Gem' is concisely described and invariably accompanied by a line drawing or photographs. With 40 contributors the quality of the description and photographs tend to vary somewhat. Many of them are woefully inadequate in detail and only confirm that surgery can only be learnt by apprenticeship in the theatre and not from an instruction manual. However, the book is worth getting a sight of to pick a few gems of low carat value.

S.I.WHITE

British Journal of Dermatology (1990) **122**, 576.

News and Notices

Dermatopathology Self-Assessment Workshop
Cleveland, Ohio, 6 October 1990

A Dermatopathology Self-Assessment Workshop will be held at the Cleveland Clinic Foundation on 6 October 1990. For further information contact Wilma F.Bergfeld MD, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, Ohio 44106, U.S.A.

Dermatology Nurses Support Group Study Day
20 September 1990

A Study Day has been organized for nurses with a full-time commitment to dermatology and will be held at the Sunderland Polytechnic on Thursday 20 September 1990. The speakers will include Professor J.A.A.Hunter and Dr W.J.Cunliffe.

The cost will be £25.00 and limited overnight accommodation at The Royal Infirmary, Sunderland, can be arranged. Further details can be obtained from Dr T.C.Hindson, Dermatology Department, The Royal Infirmary, Sunderland SR2 7JE.

Society for Pediatric Dermatology

Applications are invited for a $5000–10,000 grant for clinical or basic research in pediatric dermatology. Applications must be received by 1 May 1990.

The society for Pediatric Dermatology announces its call for papers by 1 May 1990. An annual award will be given to a pediatric or dermatology resident for a manuscript on the results of a clinical or laboratory research project related to pediatric dermatology, to be presented at the annual meeting for the Society for Pediatric Dermatology, San Diego, California, 8–11 August 1990. The award is $500 and expenses to attend the meeting and present the paper.

Applications for the research grant and manuscripts for the residents' award should be submitted to Dr Anne W. Lucky, Chairman, Committee on Awards and Goals, Society for Pediatric Dermatology, c/o Dermatology Associates for Cincinnati Inc, 7691 Five Mile Road, Cincinnati, Ohio 45230.

International Symposium on New Trends in Allergy III
Munich, 13–15 July 1990

An International Symposium on New Trends in Allergy will be held at the Cultural Center in Munich on 13–15 July 1990. For further details, contact Professor Dr Johannes Ring, Dermatologische Klinik u Poliklinik, der Ludwig-Maximilians-Universitat Munchen, Frauenlobstrasse 9–11, D-8000 Munich 2, West Germany.

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Epidermolysis bullosa acquisita antigen and the carboxy terminus of type VII collagen have a common immunolocalization to anchoring fibrils and lamina densa of basement membrane

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SUMMARY

In this study a variety of immunoelectron microscopic methods were used to define the precise ultrastructural binding site of epidermolysis bullosa acquisita antibodies (EBA-Ab). We used two EBA sera which immunoblotted with the same skin-extracted protein as that labelled by a monoclonal antibody (LH7.2) which is known to react with the carboxy terminus of type VII collagen. Gold-conjugated antibodies were used in two different immunoelectron microscopic procedures to compare the labelling characteristics of EBA-Ab and LH7.2 in normal human skin. Antibody incubations were performed using ultra-thin cryosections of unfixed skin and thin slices of fresh skin (*en bloc* technique) before conventional fixation and embedding in Epon. Both methods showed similar labelling features for both EBA-Ab and LH7.2. With ultra-thin cryosections there was labelling of the lamina densa and an undefined component of the sub-lamina densa region. With the *en bloc* technique, labelling of dermal ends of anchoring fibrils and of amorphous material recently defined as 'anchoring plaques' was evident. There was no labelling of the central banded portions of anchoring fibrils. We conclude that EBA-Ag is localized to the dermal ends of anchoring fibrils in addition to the lamina densa and possibly anchoring plaques, and thus has the same distribution as the carboxy terminus of type VII collagen.

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Epidermolysis bullosa acquisita (EBA) is an acquired blistering disease in which autoantibodies develop against the epidermal basement membrane zone (BMZ).[1] The target antigen of the autoantibodies, known as EBA antigen (EBA-Ag), is synthesized by both human keratinocytes and fibroblasts.[2,3] EBA-Ag has been found in extracts of skin basement membrane by Western blot analysis to consist of a protein of 250–290 kDa, with collagenous and non-collagenous domains.[4-7] More recently, it has been shown that EBA-Ag corresponds to the carboxy terminus (c-terminus), a 145 kDa globular domain of type VII procollagen.[8,9] Type VII collagen is also known to be a major component of anchoring fibrils, which are short curved structures forming part of the sublamina densa fibroreticular layer of the basement membrane.[10-16] They are thought to have a role in dermo-epidermal adherence and have been found to be numerically deficient or structurally abnormal in different types of the genetic mechanobullous disease, epidermolysis bullosa dystrophica.[17-20] Anchoring fibrils have a central banded portion which corresponds to the collagenous domain of the type VII collagen molecule, and a peripheral non-banded filamentous portion, which contains the non-collagenous globular c-terminus.[13-16] The dermal ends of anchoring fibrils have recently been shown to be associated with small amorphous bodies known as anchoring plaques, which contain both type IV and type VII collagen.[16] EBA-Ab has been shown by indirect immunofluorescence to bind to the dermal side of 1 M NaCl separated skin[21] and by indirect immunoperoxidase immunoelectron microscopy (IEM) to form deposits on and just beneath the lamina densa.[4,22-25] The deposition of immunoperoxidase reaction products obscure the underlying anchoring fibrils and thus do not allow the precise immuno-localization to be determined. Therefore, evidence is so far lacking that EBA-Ab specifically binds to anchoring fibrils and perhaps to the related anchoring plaques, owing possibly to the limitations of the IEM techniques that were formerly employed.

Since certain IEM techniques which use colloidal gold as a label have the potential advantage over immunoperoxidase of being able to reveal Ab binding without obscuring the related ultrastructural features, we have applied two different techniques in the present study to elucidate the exact immunolocalization of EBA-Ag in normal human skin. For comparative purposes, a parallel study was performed using a monoclonal antibody previously found to react with the c-terminus of type VII collagen.[26,27]

METHODS

Antibodies

EBA sera. Sera were used from two patients with EBA in whom the diagnosis was based on typical clinical, histological and immunopathological features of this condition.[1,22,24] Direct immunofluorescence revealed IgG deposition in the BMZ of the perilesional skin. Indirect immunofluorescence showed that the sera contained IgG antibodies which bound to the BMZ in cryostat sections of normal human skin, with titres of 1 : 160 and 1 :640. IgG from both sera bound to the dermal side of 1 M NaCl separated skin.[21] Conventional pre-embedding immunoperoxidase IEM[28] showed that both EBA-Abs bound to the lamina densa and sublamina densa zone of normal human skin. Normal human sera were used as controls.

LH7.2 monoclonal antibody. This is a mouse monoclonal antibody to the c-terminus of type VII collagen.[26,27] LH7.2 antigen is localized to the BMZ of squamous epithelia. Indirect immunofluorescence confirmed that LH7.2 bound to the BMZ in cryostat sections of normal human skin and to the dermal side of 1 M NaCl separated skin.[21] Conventional pre-embedding immunoperoxidase IEM[28] showed that LH7.2 bound to the lamina densa and sublamina densa zone of normal human skin, similarly to the EBA-Ab.

Immunoblotting. The techniques used for extracting and analysing skin BMZ proteins and for characterizing the EBA sera have been previously published.[7] Briefly, human skin was incubated in 1 M NaCl containing 50 μM phenylmethylsulphonylfluoride at 4°C for 72 h. BMZ preparations were made from epidermal and dermal fragments by incubating them in 8 M urea containing 0·3 M 2-mercaptoethanol and 50 μM phenylmethylsulphonylfluoride. The extracted proteins were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto nitrocellulose. Both the EBA sera and LH7.2 reacted with a protein of 250 kDa from the dermal preparation. After collagenase digestion (cleaving the collagenous region of type VII collagen, leaving the c-terminus intact), both EBA-Ab and LH7.2 detected a 145 kDa protein, corresponding to the c-terminus of type VII collagen.

Immunoelectron microscopy

Normal adult human skin freshly obtained from two different sites (abdomen and arm) was used as a substrate.

Labelling of cryoultrathin sections. The skin was immediately cut into small pieces (< 1 mm^3) and cryoprotected in 20% glycerol/phosphate-buffered saline (PBS), pH7·4 at 4°C for 1 h. The skin was then cryofixed by plunging into liquid propane, cooled to -190°C, at a speed of 3 m s^{-1} using a KF-80 cryofixation apparatus (Reichert-Jung, Cambridge Instruments, Cambridge, U.K.). Cryoultrathin sections (about 100 nm thick) were cut at -100°C using an FC-4D cryoultramicrotome (Reichert-Jung, Cambridge Instruments, Cambridge, U.K.) and collected on pioloform-coated nickel grids using 2·3 M sucrose, at room temperature (RT).[29] The grids were transferred to a drop of PBS and placed on a drop of PBS/1% bovine serum albumin (BSA)/5% normal goat serum (NGS) at pH 7·4 for 15 min. The specimens were then incubated in primary antibody (EBA-Ab diluted at 1 : 10 with PBS/0·1% BSA/5% NGS or neat LH7.2) at RT for 30 min. Following a rinse in PBS for 20 min at RT, the specimens were further washed in 20 mM Tris/0·9% NaCl/1% BSA at pH 8·0. They were then incubated with 5 nm colloidal-gold conjugated goat anti-human IgG (for EBA-Ab) or goat anti-mouse IgG (for LH7.2) (Janssen Life Sciences, Wantage, Oxford, U.K.) diluted at 1 : 20 in 20 mM Tris/0·9% NaCl/1% BSA at pH 8·0 for 30 min at RT. After further extensive washing in buffer and double distilled water, the sections were stained with 0·2% uranyl acetate, carbon coated and examined in a JEOL 100CX transmission electron microscope (TEM).

Pre-embedding en bloc labelling. Skin was cut into very small pieces ($< 0·5$ mm^3) and washed in PBS for 2 h on a rotary mixer at 4°C. The specimens were then incubated in primary antibody (EBA-Ab diluted at 1 : 2 with PBS/1% BSA or neat LH7.2) at 4°C for 12 h. Following a rinse in PBS for 6 h at 4°C the specimens were incubated with 5 nm colloidal-gold conjugated goat anti-human IgG (for EBA-Ab) or goat anti-mouse IgG (for LH7.2) diluted at 1 : 2 in 20 mM Tris/0·9% NaCl/1% BSA at pH 8·0 for 5 h at 4°C. After washing in PBS for 12 h as above, the specimens were fixed with half-strength Karnovsky fixative[30] for 30 min, and post-fixed with 1% OsO_4 in distilled water for 30 min at RT. After dehydration in a graded series of ethanol, the specimens were embedded in Epon 812. Ultra-thin sections were cut from the surface of the specimen and stained with 5% uranyl acetate and lead citrate, and observed in the TEM.

Control experiments. Normal human serum was used as a control for the EBA-Ab, in equivalent dilutions, for both methods. Similarly, conditioned medium from the myeloma cell line SP2/0-Ag 14 was used as control for LH7.2.

RESULTS

Both EBA-Abs obtained from different patients showed similar immunolabelling with each technique.

Labelling of cryoultrathin sections

Labelling of EBA-Ag was relatively heavy throughout the lamina densa and appeared in small clusters in the sublamina densa region (Fig. 1a). LH7.2 immunolabelling was similarly distributed over the lamina densa (Fig. 1b). Neither the lamina lucida nor the anchoring fibrils could be clearly recognized using this technique.

Pre-embedding en bloc labelling

The ultrastructure of the BMZ including anchoring fibrils and lamina densa was generally well demonstrated (Figs 2a–d; 3a–c). EBA-Ab labelled the lower surface of the lamina densa and the dermal ends of anchoring fibrils, but not the central banded portion of anchoring fibrils. Labelling of the lower surface of the lamina densa was not always related to the anchoring fibrils (Fig. 2a–d). LH7.2 showed similar immunolabelling to that for EBA-Ab, although the density of the gold particles at the dermal ends of anchoring fibrils was less than it was with EBA-Ab. The lower surface of the lamina densa was similarly decorated with gold particles (Fig. 3a–e). With both EBA-Ab and LH7.2 there was labelling of amorphous material within the upper dermis (Figs 2d, 3b,e). This was seen either as a strand or loop of material continuous with the lamina densa (Fig. 3e), or as an apparently separate structure (Figs. 2d, 3b), possibly corresponding to an 'anchoring plaque'.[16]

Controls

No specific immunolabelling was observed when normal human serum or conditioned medium from myeloma cell line SP2/0-Ag 14 was used instead of either of the primary antibodies.

DISCUSSION

This study shows conclusively, for the first time that EBA-Ag occurs in the dermal ends of anchoring fibrils, and confirms earlier reports that it is a normal constituent of the lamina densa of the epidermal BMZ.[4,22–25] Recent biochemical studies have indicated that EBA-Ag corresponds to the c-terminus of type VII collagen,[8] but previous immunoelectron microscopic studies have not shown the precise localization of the antigen. Immunoblotting data showed that the two EBA sera used in our study reacted with the same protein as did LH7.2 monoclonal Ab which previously was found to bind specifically to the non-collagenous globular domain or c-terminus of the type VII molecule.[26,27] Recent studies using a monoclonal Ab specifically recognizing the c-terminal ends of type VII procollagen dimers[13,16] have shown similar results to those of the present study. Using the *en bloc* pre-embedding technique, gold labelling was seen over the dermal ends of anchoring fibrils and associated 'amorphous patches' or 'anchoring plaques'.[16] In the present study, using the *en bloc* method we have found similar labelling sites

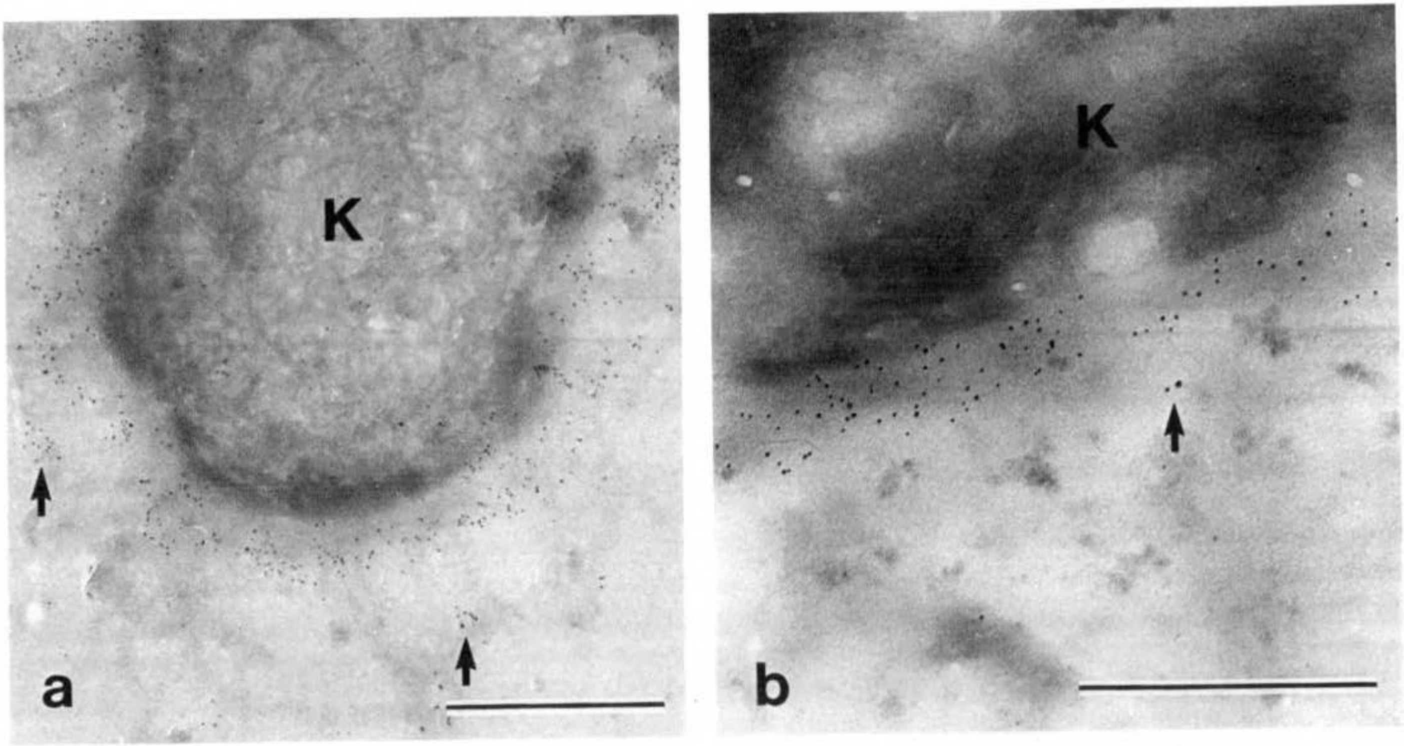

FIGURE I. Labelling on cryoultrathin sections of normal skin. (a) Epidermolysis bullosa acquisita antigen. Gold particles are localized mainly over lamina densa of epidermal basement membrane. Some labelling is present in clusters in sub-lamina densa zone (arrows); (b) c-terminus of Type VII collagen labelling is in a similar distribution to that shown in (a). K = basal keratinocyte. Bars = 5 μm.

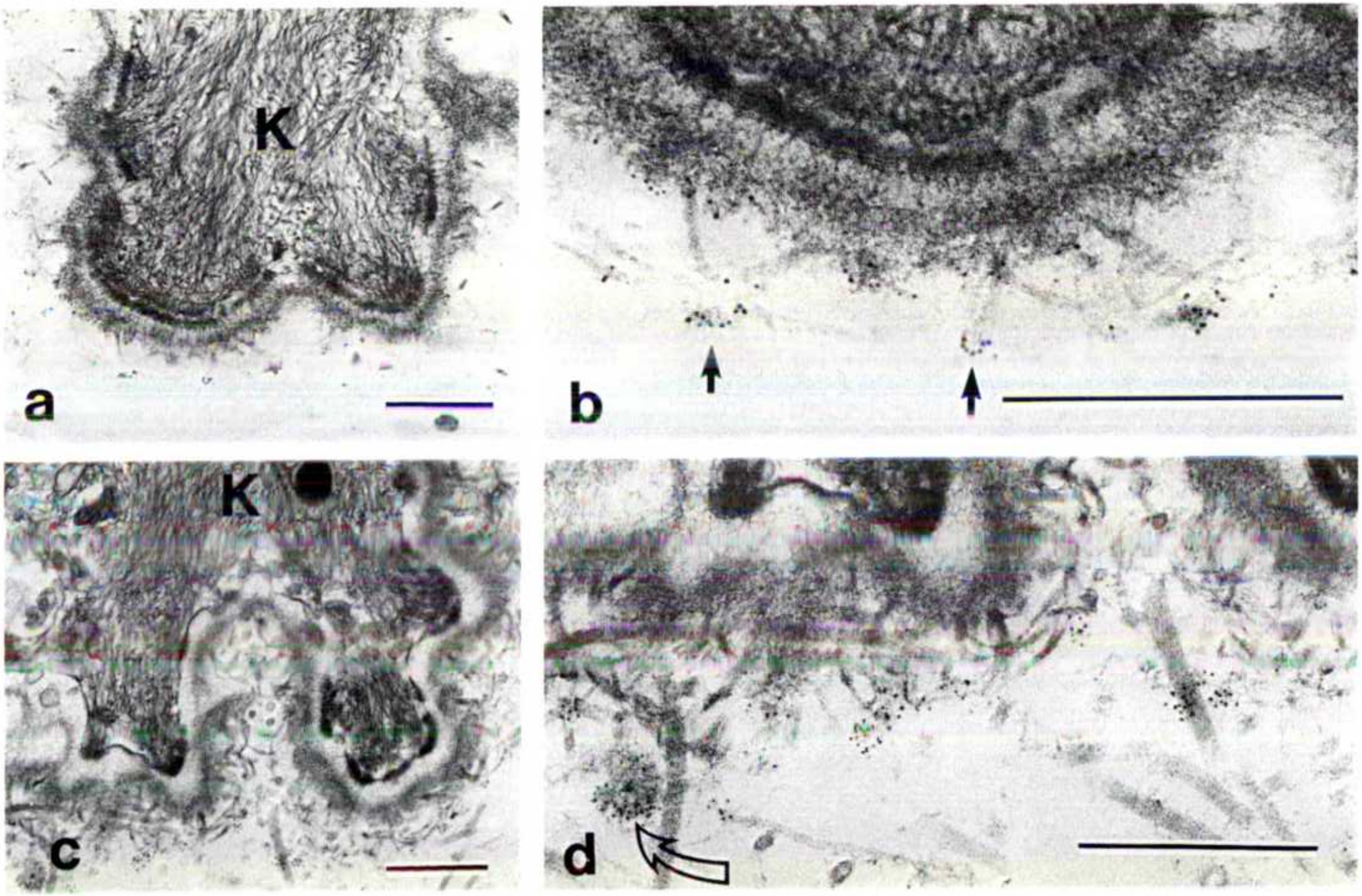

FIGURE 2. Labelling of epidermolysis bullosa acquisita antigen using the *en bloc* technique. (a), (c) Low magnification views of dermal–epidermal junction showing gold labelling related to anchoring fibrils; (b), (d) Higher magnification views of parts of (a) and (c) indicating the distribution of gold particles on the lower surface of the lamina densa and the dermal ends of anchoring fibrils (arrows) but not on the central banded portions of anchoring fibrils. In (d), there is labelling of amorphous material (open arrow) within the upper dermis, possibly corresponding to an 'anchoring plaque'. K = basal keratinocytes. Bars = 5 μm.

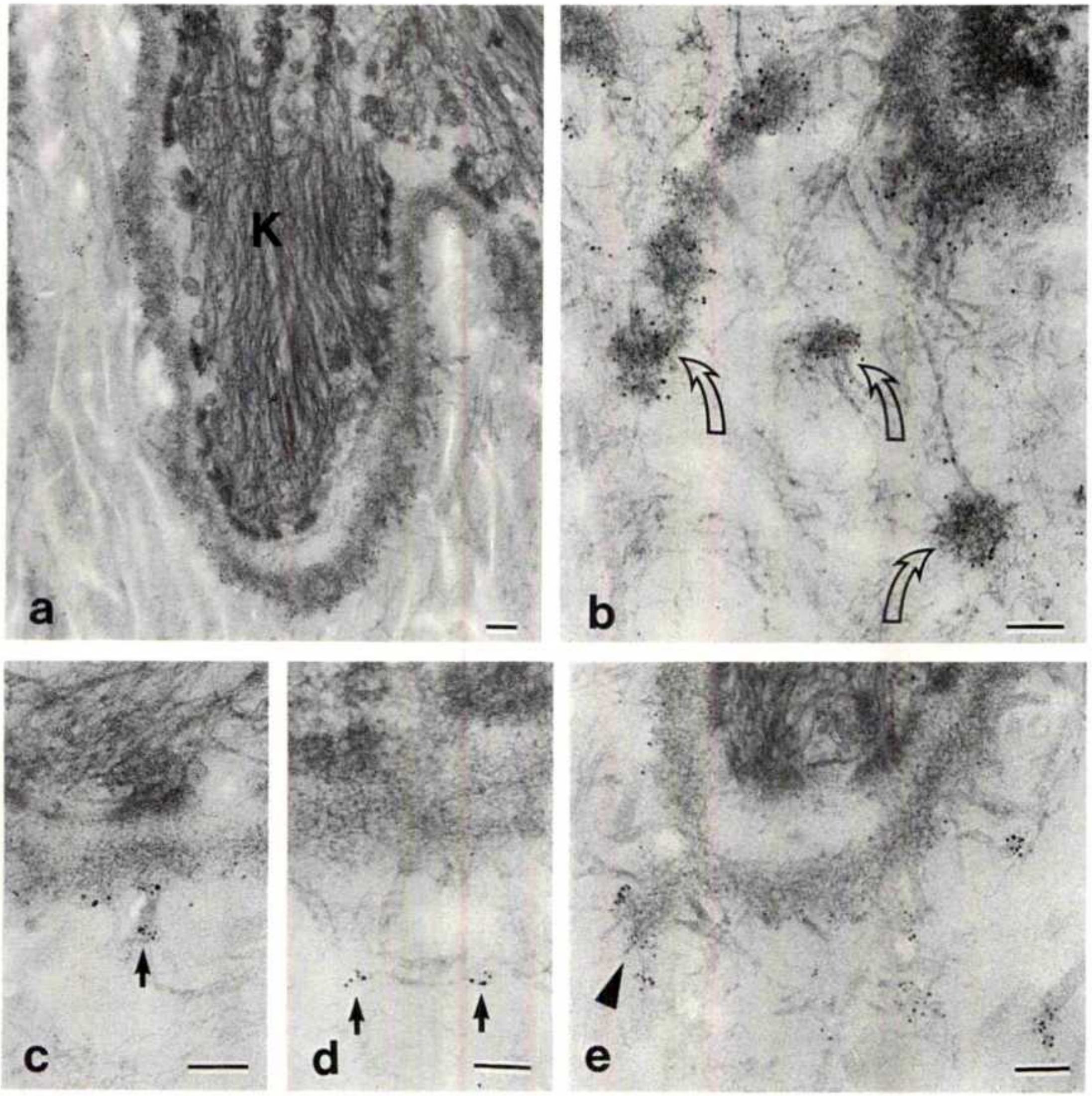

FIGURE 3. Labelling with monoclonal antibody to c-terminus of type VII collagen using *en bloc* technique. (a) Low magnification view showing labelling is mainly evident over the lamina densa and sub-lamina densa zone. (b)–(e) Higher magnification views of epidermal basement membrane zone showing labelling on the lamina densa and ends of anchoring fibrils (arrows). In (b) there is labelling of amorphous material which appears as a broken strand or in isolated patches (open arrows), apparently not continuous with the lamina densa. In (e) the labelled material (arrow head) is connected to the lamina densa. K = basal keratinocytes. Bars = 1 μm.

on the dermal ends of anchoring fibrils with both EBA-Ab (Fig. 2a–d) and anti-type VII monoclonal Ab (Fig. 3a–e). We also observed labelling of amorphous material which in some sections was apparently isolated from the basement membrane (Figs 2d, 3b) and in others, continuous with it (Fig. 3e). Studies of serial sections were not performed to determine whether or not these amorphous bodies were separated from the lamina densa.

EBA-Ab and LH7.2 labelled the lamina densa and sublamina densa region in cryoultrathin sections of unfixed tissue. However, with this method, we have been unable to show specific labelling of anchoring fibrils. In another study by some of our group,[27] using surface labelling on Lowicryl sections, the epitope recognized by LH7.2 was also largely confined to the lamina densa.

The differences between the methods used can be explained by variations in access of the antibodies to the epitopes, with possibly a greater availability of lamina densa in the surface labelling in the cryoultrathin sections, but with better preservation and/or visualization of anchoring fibrils in the pre-embedding '*en bloc*' labelling experiments.

The role of EBA-Ag and therefore of anchoring fibrils and anchoring plaques in the pathogenesis of EBA is far from clear. Binding between EBA-Ag and Ab may trigger a reaction causing release of proteolytic enzymes from the skin or infiltrating neutrophils, which in turn degrade BMZ related components and lead to sublamina densa or lamina lucida blister formation.[31] Alternatively, autoantibody deposition may directly affect the affinity between type VII collagen and other macromolecules of the BMZ[8] and perhaps disrupt the relationship between anchoring fibrils, lamina densa and anchoring plaques.

ACKNOWLEDGMENT

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Immunomodulating cytokines in atopic dermatitis and psoriasis: production of tumour necrosis factor and lymphotoxin by mononuclear cells *in vitro*

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SUMMARY

The immunomodulating cytokines, tumour necrosis factor/cachectin (TNF) and lymphotoxin (LT) are thought to play an essential role as mediators of inflammatory reactions. To evaluate the role of TNF and LT in atopic dermatitis (AD) and psoriasis, we investigated their production by mononuclear cells (MNC) *in vitro*. The 24-h supernatants of lipopolysaccharide (LPS)- and phytohaemagglutinin (PHA)-stimulated and unstimulated MNC from 26 patients with AD and 20 with psoriasis and from 17 non-atopic healthy controls were tested for the concentrations of TNF and LT using an ELISA technique. In patients with AD, TNF levels were significantly decreased in the supernatant of PHA-stimulated ($P \leq 0{\cdot}005$) and LPS-stimulated ($P \leq 0{\cdot}02$) MNC in comparison to controls. There was no significant difference in TNF production between psoriatic patients and the control group. Release of LT in the supernatant of PHA-stimulated MNC by patients and controls did not differ significantly. There was no significant spontaneous production of TNF and LT by MNC of patients and controls. These studies indicate that different immunomodulating mechanisms are responsible for triggering the inflammatory response in AD and psoriasis.

The cytokines, tumour necrosis factor/cachectin (TNF) and lymphotoxin (LT),[1,2] are thought to play an essential role as mediators of inflammatory reactions. TNF was shown to produce a broad range of regulatory and inhibitory effects on various cell types,[3–7] and to be a signal for the production of interleukin 1 (IL-1) both *in vitro* and *in vivo*.[8–9]

Atopic dermatitis (AD) and psoriasis are inflammatory skin disorders, with immunological changes.[10–22] Significant differences between these two diseases could be demonstrated with respect to the release of immunomodulating cytokines *in vitro*. Mononuclear cells (MNC) of patients with AD, in contrast to those of psoriatic patients, released decreased amounts of IL-1

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and IL-2 following stimulation.[15] Production of IL-6 by MNC on stimulation was not altered in either disease,[17] whereas unstimulated MNC of patients with psoriasis produced significantly increased levels of IL-6 compared with those from controls and from patients with AD. The *in vitro* production of interferon-α and -γ was only significantly decreased in patients with psoriasis.[18,19]

It was recently demonstrated that human epidermal cells are able to produce and to release TNF in significant amounts.[23] To evaluate the role of TNF and LT as mediators of the inflammatory response in AD and psoriasis, the production of TNF and LT by MNC was examined *in vitro* in patients with AD and psoriasis.

METHODS

Patients and controls

The AD group consisted of 26 patients (11 male, 15 female; age range 18–81 years, mean age 28 years) with moderate to severe disease and with involvement of 5–50% of the body surface area[24] (mean involvement 15 ± 3%). All patients had excoriated skin lesions with intense pruritus. The mean IgE level was 1154 kU/l (log IgE 3·06235 ± 0·11086 kU/l). The diagnosis was made according to the criteria of Hanifin and Rajka.[25] The group with psoriasis consisted of 20 patients (12 male, eight female; age range 16–81 years, mean age 49 years). They had plaque-type psoriasis involving 3–90% of the body surface area (mean involvement 29 ± 6%). None of the patients had psoriatic arthritis. The control group consisted of 17 healthy blood donors (13 male, four female; age range 22–34 years, mean age 25 years) in whom atopy was excluded from the history and the laboratory findings. Neither the controls nor the patients had received local and systemic steroid therapy or UV therapy in the 3 weeks prior to taking the blood samples.

Isolation of mononuclear cells and generation of supernatants

Unfractionated MNC were isolated from heparinized (50 U/ml) peripheral blood of patients and controls as previously described.[26] MNC were in a concentration of 5×10^6/ml in serum-free HEPES-buffered Eagle's minimal essential medium (EMEM) containing 50 μg/ml bovine serum albumin (BSA) and were incubated in the presence of either 0·5 μg/ml lipopolysaccharide (LPS) or 5 μg/ml phytohaemagglutinin (PHA) or without stimulus at 37°C in 95% humidified air. The absolute number of monocytes in MNC preparations of patients and controls was determined routinely by non-specific esterase staining (Technikon, Bad Vilbel, F.R.G.) and no significant differences between patients and controls could be detected. After 24 h the supernatants were collected, centrifuged, filtered and then stored at −70°C until tested.

Quantitation of TNF and LT

The concentrations of TNF and LT in the supernatant of patients and controls were determined using an ELISA technique. Immunoassay plates were coated overnight at 4°C with 5 μg/ml monoclonal antibody mAk199 (TNF) or mAk9b9 (LT). Non-specific binding was blocked by adding 1% BSA in phosphate-buffered saline (PBS) for 30 min at room temperature. After four washes with PBS containing 0·1% BSA and 0·05% Tween-20, supernatant was added to the wells and incubated for 4 h at room temperature. The plates were then washed four times and biotinylated monoclonal antibody mAk195 and a LT-specific polyclonal rabbit IgG, both recognizing different epitopes, were added and incubated for 2 h at room temperature. After washing, streptavidin–peroxidase complex was added and incubated for 30 min at room temperature. Peroxidase was measured by a colorimetric procedure using tetramethyl–

benzidine (TMB) as substrate and the reaction stopped after 15 min by adding 1 M sulphuric acid. The absorbance was measured at 450 nm using a Titertek Multiscan MCC. A standard titration curve was obtained by making serial dilutions of a known sample of recombinant human TNF or LT. The detection limit for TNF and LT was at 10 pg/ml.

Reagents and stimuli

The MAbs against recombinant human TNF (mAk 199 and mAk 195) and LT (mAk 9b9) as well as a LT-specific polyclonal rabbit IgG were obtained from Boehringer Mannheim, F.R.G. Phytohaemagglutinin (PHA) was obtained from Wellcome (Burgwedel, F.R.G.), S-form LPS from Salmonella abortus equi was a gift from Dr C. Galanos, MPI for Immunobiology, Freiburg, F.R.G. and purified BSA was from Sigma, Munich, F.R.G. Culture media were purchased from Biochrom-Seromed, Berlin, F.R.G.

Statistics

Statistical analysis of the data was carried out using the Mann–Whitney *U*-test and Spearman's rank correlation coefficient.

RESULTS

In patients with AD, the TNF levels were significantly decreased in the supernatant of PHA-stimulated ($P \leqslant 0{\cdot}005$) and LPS-stimulated ($P \leqslant 0{\cdot}02$) MNC compared to controls (Fig. 1). There was no significant difference in TNF production between psoriatic patients and controls. Release of LT in the supernatant of PHA-stimulated MNC did not differ significantly between patients and controls (Fig. 2), and upon stimulation with LPS no increase in LT production

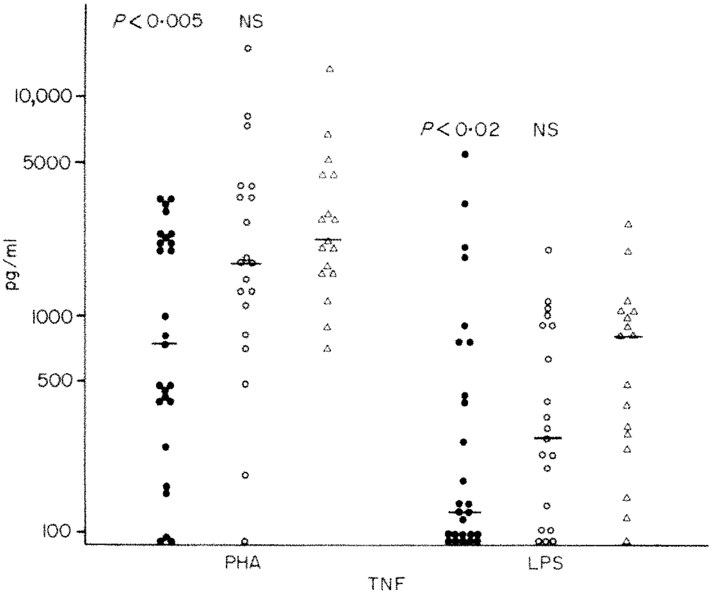

FIGURE 1. Production of TNF upon stimulation with PHA or LPS by MNC from patients with atopic dermatitis (●), patients with psoriasis (○) and from non-atopic healthy controls (△). Bars indicate medians. NS, not significant.

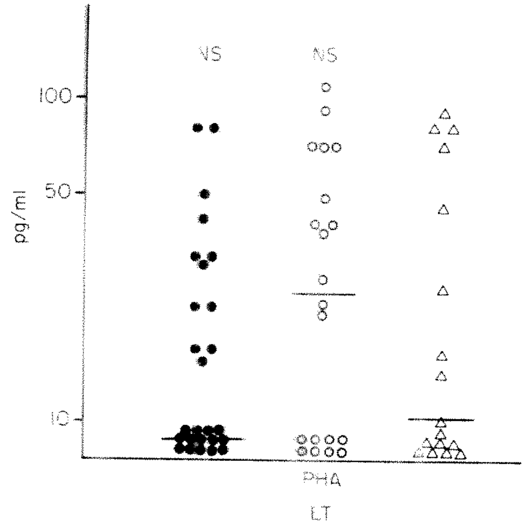

FIGURE 2. Production of LT upon stimulation with PHA by MNC from patients with atopic dermatitis (●), patients with psoriasis (○) and from non-atopic healthy controls (△). Bars indicate medians. NS, not significant.

could be detected. There was no significant spontaneous production of TNF and LT by MNC from patients and the controls. There were no significant differences in LT or TNF production between males and females in any of the three groups, or when subjects in all three groups were analysed together.

There was a significant correlation between TNF levels on stimulation with PHA, and with LPS, when all subjects were analysed together ($R=0{\cdot}64$, $P\leqslant 10^{-6}$), and when the groups were analysed separately (AD: $R=0{\cdot}63$, $P\leqslant 0{\cdot}002$; psoriasis: $R=0{\cdot}62$, $P\leqslant 0{\cdot}001$). There was also a significant correlation between TNF and LT levels following stimulation with PHA when all subjects were analysed together ($R=0{\cdot}6$, $P\leqslant 3\times 10^{-6}$), or when the groups were analysed separately (AD: $R=0{\cdot}73$, $P\leqslant 0{\cdot}0003$; psoriasis: $R=0{\cdot}46$, $P\leqslant 0{\cdot}05$). No significant correlation could be detected between age, body surface involvement or IgE levels and TNF or LT production. Only IgE levels and body surface involvement were correlated significantly ($R=0{\cdot}55$, $P\leqslant 0{\cdot}03$).

DISCUSSION

Our results show that in atopic dermatitis, TNF production by MNC *in vitro* was significantly decreased following stimulation with LPS and PHA when compared with both healthy controls and psoriatic patients. The production of IL-1 and IL-2 after stimulation had previously been reported to be decreased in AD.[16] However, this did not reflect a decreased capacity of monocytes in AD patients to release immunomodulating cytokines, since the production of IL-6[17] and interferon-α[18] appeared to be unaffected. In contrast, a decreased capacity to produce interferon-α as well as interferon-γ,[19] associated with an increased spontaneous production of IL-6,[17] could be demonstrated in psoriatic patients. It appears that there are differences in the dysregulation of the cytokine network in AD and psoriasis.

No significant spontaneous production of TNF or LT could be detected in either disease *in* *vitro*. IgE levels correlated with body surface involvement and activity. There was, however, no correlation of TNF levels with the IgE level or body surface involvement, suggesting that the decreased production of TNF in AD is not a direct reflection of the inflammatory process in the skin. However, it was reported recently that stimulation of PMN *in vitro* deactivated the cells for subsequent stimulation with the same cytokine,[7] and it may be that excessive stimulation *in vivo* could be the cause of a decreased TNF production by monocytes *in vitro*. This hypothesis is further supported by the finding that human epidermal cells are able to synthesize and release TNF upon stimulation.[23] Alternatively, the decreased production of TNF could be a sign of 'exhaustion' due to preceding activation of monocytes *in vivo*.

The potential role of monocytes in the pathogenesis of AD is supported by our finding that release of the non-monocyte product LT did not show any significant changes in AD. Although TNF and LT have similar biological activities, share a common receptor and are encoded by genes which are structurally related[3,4] LT is exclusively produced by lymphocytes,[27,28] whereas TNF can be produced by both lymphocytes and monocytes.[27,28]

As expected from previous reports,[27,28] stimulation with PHA induced the release of significant amounts of LT, whereas no spontaneous production could be detected.[28] Upon stimulation with mitogens such as PHA lymphocytes are capable of releasing both cytokines. However, PHA only induces the release of low levels of TNF by lymphocytes. LPS, which is a potent inducer of TNF production from monocytes, failed to induce significant levels of TNF in lymphocyte preparations.[27] The results indicate that monocytes are the cell population responsible for the decreased release of TNF by MNC of AD patients. In contrast, the capacity of lymphocytes to release immunomodulating cytokines, particularly interferon-γ[18] and lymphotoxin, is apparently not suppressed in AD, and there are signs of T-cell activation in AD as well as in psoriasis.[20]

The TNF concentrations found in the supernatants using our ELISA system agrees with other data on TNF production by MNC from healthy controls,[29] and with PHA as the stimulus, almost identical results were obtained. LPS-stimulation led to slightly lower TNF levels, which may be due to a different source and concentration of LPS in this study. Correlation of the amount of TNF produced with characteristics such as sex or age in patients as well as in healthy controls has not been successful.

Inflammatory processes are regulated by a number of cytokines including cytotoxins, interferons, colony-stimulating factors and interleukins. We suggest that different immunomodulating cytokines mediate the inflammatory reactions in AD and psoriasis. AD, in particular, is characterized by a significantly decreased capacity of monocytes to release immunomodulating cytokines. This defect probably involves more than one regulatory step since TNF and IL-1 production appear to be differentially modulated.[30]

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Antigen-presenting cells in the skin and placenta in pemphigoid gestationis

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SUMMARY

In pemphigoid gestationis (PG), one of the major initiating events is the aberrant expression of the class II molecules of the major histocompatibility complex in the placenta. We used a panel of 13 monoclonal antibodies to investigate the phenotype of the MHC class II positive antigen-presenting cells (APC) in the skin and placenta in PG. In the skin, the APC show reactivity with a variety of macrophage markers and the CD1 marker of Langerhans cells. By contrast, the MHC class II positive cells in the placenta showed no reactivity with macrophage or Langerhans cell markers, but were the cytokeratin-positive trophoblast or vimentin-positive stromal cells.

Pemphigoid gestationis (PG) is a rare bullous dermatosis of pregnancy that is characterized by immunoglobulin deposition and complement fixation in the cutaneous basement membrane.[1] The association with pregnancy[2] and occasionally with trophoblastic tumours[3] suggested that a placental antigen cross-reacting with skin may trigger the autoimmune response. Kelly *et al.*[4] have recently confirmed that there is cross-reactivity between skin and placenta exhibited by serum from patients with PG. The antigen has been further characterized as a basement membrane zone glycoprotein of 180 kDa.[5]

In previous studies of placental immunology in PG it has been shown that there is aberrant expression of the class II molecules of the major histocompatibility complex (MHC) in the placenta.[6,7] A similar observation has been made in target organs in other organ-specific autoimmune diseases and its significance to initiation of the autoimmune response emphasized.[8] In these organ-specific autoimmune diseases the cells expressing class II MHC molecules are the parenchymal cells which are the eventual target of the abnormal immune response.[9]

During immune responses class II MHC molecules may be expressed by a variety of cells including activated lymphocytes, Langerhans cells and other antigen-presenting cells,

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endothelium and parenchymal cells.[10] In this study we have investigated the phenotype of antigen-presenting cells in the skin and placenta of patients with PG.

METHODS

Tissues

Term placentae from five patients with clinically and immunologically defined PG were obtained following delivery. Fresh skin biopsies were available from four of the PG patients. These consisted of both lesional and perilesional skin. Skin biopsies were obtained from five normal, healthy, non-smoking volunteers with no evidence of skin disease. Blocks from term placenta from five normal pregnancies were also sampled as controls.

Small blocks of these various tissues were snap frozen and stored at −70°C until use. Cryostat sections 6 μm thick were cut and stained with haematoxylin and eosin. Multiple adjacent sections were cut and stained by immunocytochemical methods.

Immunocytochemistry

The panel of monoclonal antibodies used is summarized in Table 1. Antibody binding to tissue sections was visualized by an indirect immunoperoxidase method. Control sections were incubated with an irrelevant hybridoma supernatant (anti-AFP) replacing the monoclonal antibodies.

RESULTS

Morphological examination of the normal and PG placentae showed no major differences. However, in three of the PG placentae a few focal mononuclear cell aggregates were seen in the amniochorionic stroma.

Normal placentae

The cytotrophoblast and syncytiotrophoblast of normal placentae stained with CAM 5.2 while the stromal cells stained with vimentin. In sections stained with DA 6.231 a population of

TABLE 1. Antibodies and specificities

Antibody	Specificity	Cellular distribution
Dako T6	CD1	Langerhans cells
Dako T11	CD2	T cells
UCH T1	CD3	T-cell receptor complex
SAPU RFT4	CD4	Helper T subset
SAPU RFT8	CD8	Suppressor/cytotoxic T subset
Ki M1	CD11c	Monocyte/macrophage, Langerhans cell
FMC 17	CD14	Monocyte/macrophage, DRC
Dako M1	CD15	Monocyte/macrophage, granulocyte
Dako CD19	CD19	B cell
Dako IL2-R	CD25	IL-2 receptor
CAM 5.2	Cytokeratin	Epithelium
Vimentin (Dako)	Vimentin	Mesenchymal cells
DA6.231	MHC class II	

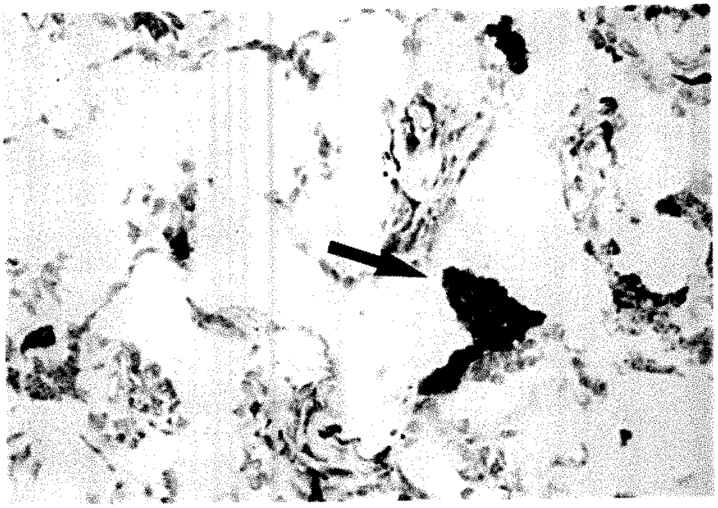

FIGURE 1. Cells in the amniochorionic stroma and in the trophoblast (arrow) show MHC class II molecule expression in PG. DA 6.231, immunoperoxidase, placenta in pemphigoid gestationis.

mononuclear cells in the amniochorionic stroma showed reactivity. No cells staining for CD2, CD3, CD4, CD8 and CD25 were seen. CD15 reactivity was seen on the endothelium of placental villus capillaries and occasional cells staining with CD15 were seen in the amniochorionic stroma. No CD1 reactivity was seen. There was CD14 staining of the endothelium of the placental blood vessels but no reactivity with this antibody was seen in the amniochorionic stroma. No CD11c or CD19 reactivity was seen.

Placentae from PG patients

The cytotrophoblast and syncytiotrophoblast stained with CAM 5.2 and the stromal cells with vimentin. Sections stained with DA 6.231 showed an abundant population of mononuclear spindle-shaped cells expressing MHC class II molecules in the amniochorionic stroma and focal staining of the trophoblast (Fig. 1). Within all of these placentae there were occasional cells staining with CD2 and CD3. In adjacent sections some of these cells showed CD4 positivity and some CD8 positivity but the cells were not sufficiently numerous to perform a quantitative analysis or CD4/CD8 ratio. A few of these cells showed CD25 reactivity. Occasional cells in the amniochorionic stroma stained with CD14 and CD15. The endothelium of placental capillaries showed CD14 and CD15 reactivity. No CD1 nor CD11c reactivity was seen.

Normal skin

No CAM 5.2 reactivity was seen but dermal fibroblasts and endothelial cells stained for vimentin. Dendritic cells within the epidermis stained with CD1, DA 6.231 and CD11c (Fig. 2). No keratinocytes stained for the MHC class II molecules. The CD11c antibody also stained dermal dendritic cells. Occasional mononuclear cells in the dermis stained with the T-cell antibodies CD2, CD3, CD4 and CD8, but no reactivity for the activation marker CD25 was seen. No CD19 nor CD15 reactivity was seen. Dermal vascular endothelium showed DA 6.231 and CD11c reactivity.

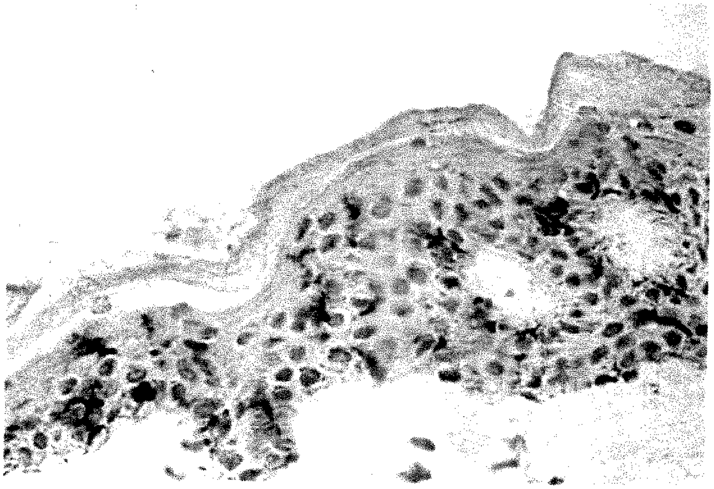

FIGURE 2. Epidermal dendritic cells in normal skin show CD1 reactivity. Dako T6, immunoperoxidase, normal skin.

Lesional and perilesional skin from pemphigoid gestationis

Examination of haematoxylin and eosin stained sections confirmed the diagnosis of PG. No CAM 5.2 reactivity was seen in the epidermis. Vimentin staining was seen in dermal fibroblasts and endothelial cells.

In all four cases the epidermal MHC class II reactivity was confined to dendritic cells with no keratinocyte reactivity (Fig. 3a). Cells of a similar dendritic morphology and distribution also stained in the adjacent sections with CD1 and CD11c (Fig. 3b). Dermal dendritic cells, inflammatory macrophages and neutrophils showed CD11c reactivity, and the macrophages and neutrophils showed CD15 positivity. Mononuclear cells within the upper dermal inflammatory infiltrate showed CD2, CD3, CD4 and some CD8 immunoreactivity, but in only two cases was CD25 reactivity seen in a few cells. A few dermal mononuclear cells showed DA 6.231 reactivity. In two cases a few cells showed CD19 reactivity. Dermal blood vessels showed DA 6.231 and CD11c reactivity.

DISCUSSION

The aberrant expression of MHC class II molecules by parenchymal cells is considered to be important in initiating the immune response in organ-specific autoimmune disease.[8] It has been shown previously that in PG there is aberrant expression of MHC class II molecules by cells in the amniochorionic stroma.[6,7] We have suggested that this is responsible for triggering the allogeneic reaction known to occur in PG[11] and that tolerance to the self basement membrane component, PG antigen, is broken because it is seen by the maternal immune system in the context of foreign MHC.[12] In the skin, antigen-presenting cells, such as Langerhans cells, show constitutive expression of MHC class II molecules,[11] as it is essential for T lymphocytes to recognize both antigen plus MHC molecules.[12]

In this study, we have identified differences in the immunophenotype of MHC class II molecule positive cells in the skin and placenta in PG. In normal skin, MHC class II molecules are expressed on the surface of intraepidermal dendritic cells (Langerhans cells), dermal

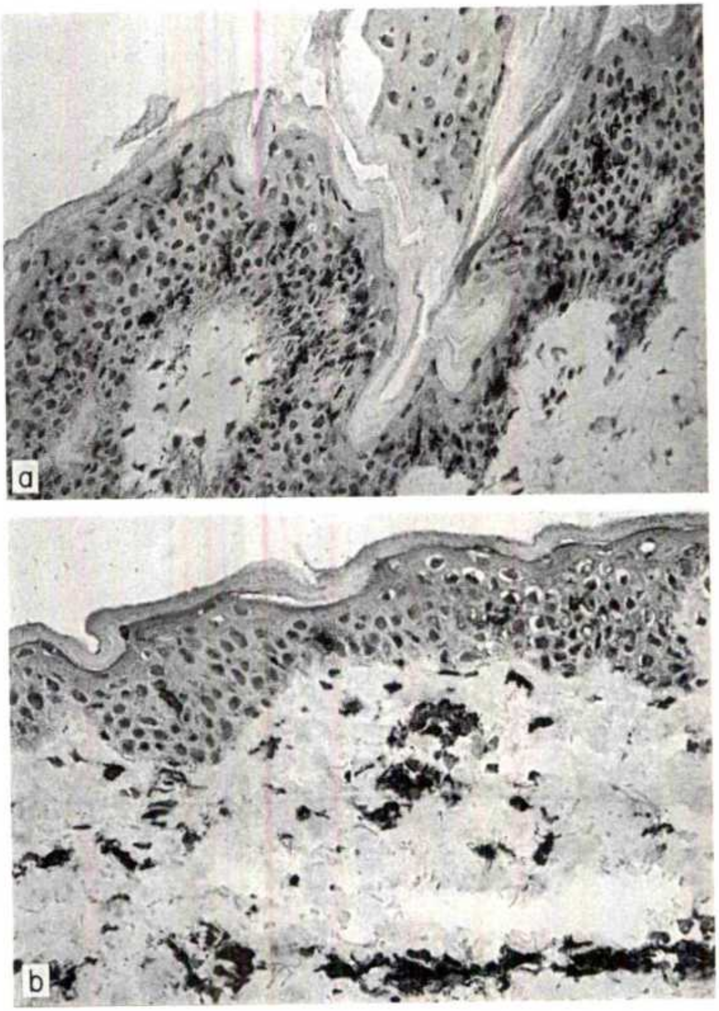

FIGURE 3. (a) In PG epidermis MHC class II expression was only seen on the epidermal dendritic cells and was not seen on keratinocytes. DA 6.231, immunoperoxidase, PG perilesional skin. (b) Epidermal dendritic cells, dermal dendrocytes and vascular endothelium express the CD 11c antigen in perilesional skin from a case of PG. KiM1, immunoperoxidase, PG perilesional skin.

dendritic cells and endothelium.[13] In lesional skin, we found MHC class II molecules on these three cell types and on some of the mononuclear cells of the inflammatory infiltrate. The Langerhans cells were identified by the surface-membrane reactivity with a CD1 monoclonal antibody.[14] These cells, dermal dendritic cells and endothelium also expressed the p150,95 C3bi receptor detected by the CD11c antibody Ki M1.[15] The dermal dendritic cells and endothelium, but not Langerhans cells, expressed the p55 antigen of CD14 reactivity.[15] No reactivity for MHC class II molecules, gp150,95 or p55 was seen on keratinocytes. In other inflammatory skin disease, particularly those characterized by lymphocytic infiltration of the epidermis, keratinocyte expression of MHC class II molecules[16,17] and other leucocyte antigens[18–20] has been described. The expression of these molecules in bullous disease has not been shown consistently.[21]

In lesions of PG, an inflammatory infiltrate composed of lymphocytes, macrophages and polymorphonuclear leucocytes is seen in the upper dermis.[2] We have confirmed the presence of these different cells in the infiltrate and have shown that some express MHC class II molecules. Cells expressing monocyte/macrophage markers such as CD11c, CD14 and CD15 are numerous. Macrophages express MHC class II molecules during inflammatory responses,[15] and cells co-expressing macrophage markers and MHC class II molecules were seen immediately underlying the epidermal–dermal junction in lesional, but not normal skin. Similar observations in other bullous lesions have been made using other macrophage markers.[19] The significance of this distribution of MHC class II positive non-Langerhans cells to antigen processing in bullous disorders is unknown.

B cells were sparse in the upper dermal infiltrate but T cells were numerous. In serial sections, T cells of a CD4 (MHC class II restricted) phenotype were more numerous than the CD8 (MHC class I restricted) phenotype. Similar findings have been seen in other organ-specific autoimmune disease, supporting the hypothesis that the expression of MHC class II molecules is important in antigen presentation in these diseases.[8,9]

By contrast to the findings in the skin, the cells in the placenta which were MHC class II positive did not express macrophage markers. In the normal placenta, spindle-shaped cells in the amniochorionic stroma express MHC class II molecules.[6,7] In term placentae from PG patients, MHC class II molecules were seen on these spindle cells, and on an additional population of polygonal mononuclear cells in the amniochorionic stroma with focal expression on the trophoblast epithelium. The stromal cells in both normal and PG placenta did not express p150,95 (CD11c) nor p55 (CD14). These spindle cells also failed to show CD1 reactivity and are clearly of a phenotype different from antigen-presenting cells in other sites including skin. Unlike lesional skin the epithelium of the placenta, the trophoblast, did show focal MHC class II expression. In the normal placentae and in placentae from two patients with linear IgA disease who became pregnant, no MHC molecules were detected on the trophoblast.[7]

The placentae at term from patients with PG contained only a very few inflammatory cells detected by leucocyte markers, despite the fact that immune complexes are deposited in the amniotic and chorionic basement membranes.[7] The deposition of immune reagents in the placentae in PG has been implicated in the placental dysfunction and impaired foetal outcome which has been shown to occur in PG.[2]

We have previously suggested that the autoimmune response in PG is initiated when the maternal immune system encounters a cutaneous autoantigen in the context of foreign, that is paternal, MHC class II molecules in the placenta.[7,12] The placental cells which express these MHC class II molecules differ immunologically from other antigen-presenting cells and have the phenotype of stromal (vimentin positive) or trophoblastic (CAM 5.2 positive) cells. The aberrant expression of MHC class II molecules by these cells in PG appears to be important in triggering the immune response against the 180 kDa PG antigen in the placenta.[5]

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Solar urticaria: studies on mechanisms of tolerance

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SUMMARY

The mechanisms by which tolerance is induced in solar urticaria were investigated in two patients whose eruptions were induced by wavelengths in the range 320–455 nm in one patient and 400–495 nm in another. Tolerance to radiation was induced by repeated exposures of the skin to the eliciting wavelengths of light. Weal and flare responses to intradermal injections of histamine and the histamine-releasing agent (codeine) were unaltered in the tolerant skin when compared with adjacent normal skin. Intradermal injection of *in vitro* irradiated serum or plasma from the patients induced an urticarial reaction in the unexposed skin but not in tolerant skin and repeated injections induced tolerance to the eliciting radiation. The results suggest that tolerance is not due to exhaustion of the photoallergen in the skin, or to an increase of the mast-cell degranulation threshold caused by exposure to ultraviolet radiation, or mast-cell mediator depletion, or histamine tachyphylaxis. It is likely that binding sites of IgE on mast cells remain occupied by the photoallergen during the state of tolerance, and that histamine release from mast cells is blocked.

In most types of physical urticaria, including solar urticaria, it is possible to induce tolerance by graduated exposure to the inducing stimulus.[1,2] Although the mechanism by which tolerance develops is unclear, one explanation could be that in solar urticaria repeated exposures to eliciting radiation produces degranulation of mast cells resulting in the exhaustion of mediator reserves. In one study of three patients rendered tolerant to UVA by repeated exposures, histamine release and mast cell degranulation, evolved by UV-irradiation, were largely prevented.[3] Weal and flare responses to injected histamine and a histamine-releasing agent (codeine) were unaltered in the tolerant skin. The authors concluded that the state of tolerance was due to an increase in the mast-cell degranulation threshold and not to mast-cell mediator depletion or histamine tachyphylaxis.

In the present study of two patients with solar urticaria, the mechanisms by which repeated

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exposures to eliciting radiation leads to tolerance were investigated. Cutaneous responses to intradermal codeine, histamine, and patients' serum or plasma activated by *in vitro* irradiation were examined in tolerant and normal skin. An attempt was made to induce tolerance to the eliciting radiation by repeated injections of serum or plasma activated *in vitro* by the appropriate wavelengths of light.

METHODS

Patients

A 49-year-old woman (case 1) and 69-year-old woman (case 2) were studied. They had a history of solar urticaria of 10 years and 3 months, respectively. There was no personal or family history of atopy and no other types of urticaria. The lesions were elicited by exposure either to direct sunlight or radiation through window glass of 5–10 min duration. The eruption faded within 2–3 h. In both patients, repeated daily exposures to sunlight induced tolerance to weal formation that lasted only for a period of 2 or 3 days when the exposures were discontinued. Physical and laboratory examinations, including blood chemistry, urine analysis and blood count with differentials were all normal. Quantitative porphyrin studies of blood and urine were normal. The serum IgE in both patients was within the normal range.

Radiation sources

Sunlamp. The source of polychromatic UVB was a bank of 10 fluorescent bulbs (Philips TL 20W/12). The emission spectrum extends from 285 to 350 nm with a maximum between 310 and 315 nm. The UVB irradiance was 2·5 mW/cm^2 at a target distance of 30 cm.

High intensity UVA. The source of polychromatic UVA was from a high-pressure metal-halide lamp (UVASUN 3000, Mutzhas, Munich, F.R.G.) that emits wavelengths between 330 nm and 460 nm without any measurable UVB.[4] The UVA irradiance was 66 mW/cm^2 at a target distance of 30 cm.

Infra-red. Infra-red radiation was provided by a 300 W Sollux lamp with red filter (Sollux 760, Heraeus, Hanau, F.R.G.). It emits between 600 and 3000 nm. The irradiance was 101 mW/cm^2 at a target distance of 35 cm.

Solar simulator. Solar simulating polychromatic UV-radiation and visible light were obtained from a high-pressure xenon arc (Cermax, ELC Technology, Sunnyvale, CA, U.S.A.). Several detachable cut-off glass filters (GG 400, GG 420, GG 455, GG 475, GG 495, OG 515, Jenaer Glaswerk Schott, Mainz, F.R.G.) were used in combination with the solar simulator. The measured irradiance at the solar simulator exit was 150 mW/cm^2.

Dosimetry. For dosimetry of the filtered solar simulator and the infra-red lamp a thermopile attached to a digital wattmeter (Müller, Moosinning, F.R.G.) was used. A UV-meter with separate UV-detectors for UVA and UVB (Centra UV-Messgerat, Osram, Munich, F.R.G.) served to determine the UV-irradiance of the UVASUN and the UV 800.

Action spectrum studies

Small test sites (1 cm^2) on the back, lateral aspect of the upper arms or ventral aspects of the forearms were exposed to various doses of broad-band UVB, UVA and infra-red as well as radiation from a solar simulator filtered with several cut-off glass filters. Thus, the diagnosis was confirmed and the causative wavelengths and minimal exposure doses required to induce wealing (minimal wealing dose (MWD)) were determined.

In vitro irradiation of serum and plasma

Freshly separated samples of serum and/or plasma from the patients were placed in a Petri dish and exposed directly to 2 MWDs of eliciting radiation. Immediately after, 0·1 ml aliquots of the patients' own serum and/or plasma, both irradiated and non-irradiated, were intradermally injected into the flexor surface of the forearm.

Induction of tolerance

An attempt was made to induce tolerance by repeated exposures with eliciting radiation. UVA or visible light was used in case 1 and case 2, respectively. Areas of 1 cm^2 on the patient's back

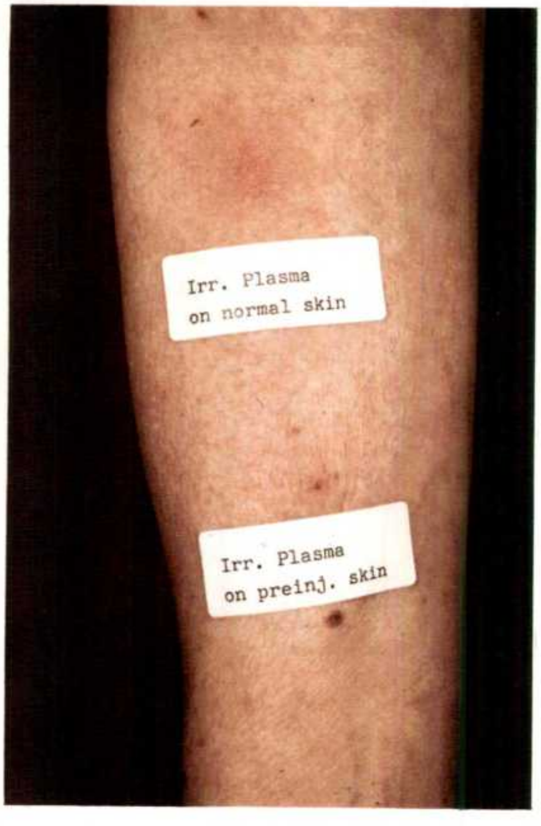

FIGURE 1. Injection of irradiated plasma induced weal and flare in normal skin (above) but not in skin tolerant due to repeated injections of irradiated plasma (below).

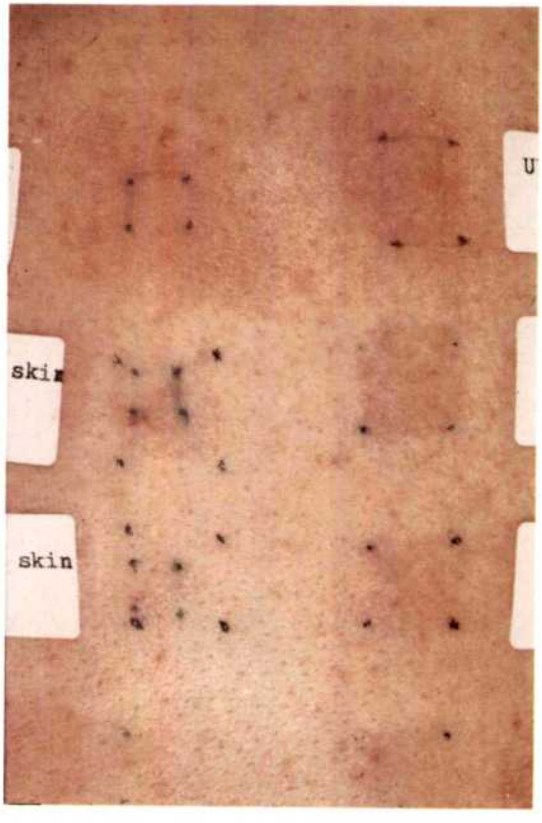

FIGURE 2. Irradiation with UVA induced weal and flare in control sites (above) but not in sites pretreated by repeated injections of irradiated plasma (left, middle and below). Pre-irradiation with UVA also rendered the skin tolerant with little erythema observed (right, middle and below).

were exposed to 2 MWDs of eliciting radiation. When wealing and erythema had subsided, 2–3 h later, the same sites were exposed to eliciting radiation again.

Studies on the mechanism of tolerance

A volume (0·1 ml) of the patients' *in vitro*-irradiated serum or plasma, 0·035% codeine phosphate, and 0·001% histamine in physiological saline solution were injected intradermally into separate sites where tolerance had been induced by repeated exposures to eliciting radiation, and also into normal skin.

Serum or plasma (0·1 ml) from the patient was *in vitro*-irradiated and intradermally injected into the skin of the back of the same patient, and equal amounts of the irradiated serum or plasma were injected into the same sites 1 and 2 h later. Twenty-four hours after the last injection, the test sites and contralateral control sites were exposed to 2 MWDs of eliciting radiation.

RESULTS

Action spectrum studies

Case 1. The action spectrum ranged from 320–455 nm. Phototesting with UVB was negative. Wealing was induced by irradiation with the solar simulator using GG 400 or GG 420 filters. Irradiation with the solar simulator plus GG 455 or GG 475 was negative. The MWD of polychromatic UVA (UVASUN 3000, 330–460 nm) and of visible light (solar simulator plus WG 420) was 2 and 10 J/m^2, respectively.

Case 2. The action spectrum ranged from 400 to 495 nm. Phototesting with UVA and UVB was negative. Wealing was induced by irradiation with the solar simulator in combination with each of the filters GG 400, GG 420, GG 455 or GG 475. Irradiation with the solar simulator with GG 495 or OG 515 filters was negative. The MWD of visible light obtained from the solar simulator filtered with GG 420 was 40 J/cm^2.

TABLE 1. Experimental proof for proposed mechanism of tolerance

Mode of inducing tolerance	Type of challenge	Result
Eliciting radiation	Histamine	Urticaria
Eliciting radiation	Codeine	Urticaria
Eliciting radiation	*In vitro* irradiated patients' plasma	No urticaria
None (control)	*In vitro* irradiated patients' plasma (Fig. 1)	Urticaria
In vitro irradiated patients' plasma	*In vitro* irradiated patients' plasma (Fig. 1)	No urticaria
In vitro irradiated patients' plasma	Eliciting radiation (Fig. 2)	No urticaria
In vitro irradiated patients' plasma	Codeine	Urticaria

In vitro irradiation of serum and plasma

After 20 min, irradiated serum produced a weal surrounded by erythema in case 1. In case 2, only the irradiated plasma and not the irradiated serum induced a positive reaction. Non-irradiated serum and/or plasma yielded negative results. This examination was repeated twice on the skin of the back of both patients, with similar results.

Induction of tolerance

Three exposures were required until the skin became tolerant. Tolerance lasted for 3 days and on day 4 weal and flare were the same as before.

Studies on the mechanism of tolerance

Injection of irradiated serum or plasma into unexposed skin produced a definite weal surrounded by erythema within 30 min. Injection of irradiated serum or plasma into tolerant skin led to a barely perceptible weal, which subsided 30 min after injection. Both tolerant skin and unexposed skin, however, demonstrated the same reaction to injection of histamine or codeine.

The first injection of irradiated serum or plasma into unexposed skin was followed by a prominent weal and flare. The second injection into the same site caused a somewhat smaller weal. No detectable weal and flare followed the third injection (Fig. 1). Irradiation 24 h later with eliciting wavelengths induced wealing and flare in control sites but neither erythema nor wealing was found at the sites where serum or plasma was injected (Fig. 2). Injection of 0·035% codeine phosphate, however, induced definite urticarial weals at these test sites. The results of our experiments are summarized in Table 1.

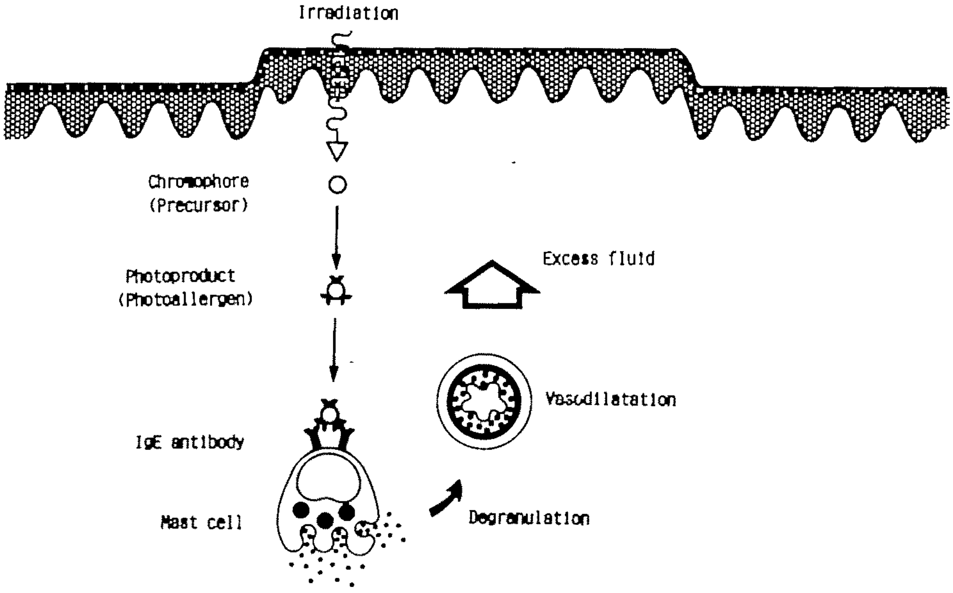

FIGURE 3. Pathomechanism of solar urticaria.

DISCUSSION

It has been assumed that irradiation of the patients' serum or plasma with eliciting radiation produces a photoproduct which induces a weal and flare reaction. As suggested by Horio,[5] this might act as a photoallergen inducing an immediate type hypersensitivity reaction. Thus, solar urticaria may develop in the manner outlined in Figure 3. A photoallergen is formed from a precursor in the patient's skin by irradiation. An IgE-mediated hypersensitivity to the photoallergen then develops with binding of specific IgE to mast cells in the skin. Subsequent irradiation of the skin elicits an allergic reaction of the immediate type with a weal and flare resulting from the release of histamine from mast cells.

From this hypothesis we have tried to explain the mechanism by which tolerance is induced. In this study, as in that of Keahey *et al.*,[3] weal and flare responses to injected histamine and codeine were unaltered in tolerant skin compared to the normal skin. Keahey *et al.*[3] showed on electron microscopy intact mast-cell granules in the tolerant skin and we have confirmed this observation in another patient.[6] These findings suggest that mast cell mediator depletion and histamine tachyphylaxis are not responsible for the inhibition of solar urticaria. *In vitro*-irradiated plasma containing the photoallergen was found to produce an urticarial reaction in the unexposed skin of the patients but failed to produce a reaction at sites tolerant to radiation, indicating that tolerance is not due to exhaustion of the photoallergen in the skin. In addition, we were able to demonstrate that repeated injections of serum or plasma activated by *in vitro* irradiation also induced tolerance to eliciting radiation but, again, weal and flare responses to intradermal codeine were unaltered in tolerant skin. This indicates that elevation of the mast-cell degranulation threshold due to direct exposure of mast cells to UV-irradiation can be ruled out as the underlying mechanism of tolerance. It is likely that the binding sites of IgE on mast cells remain occupied by the photoallergen during the state of tolerance and IgE-mediated histamine release from mast cells is blocked.

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Mechanical properties of human forearm and vulvar skin

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SUMMARY

Using a newly developed suction device, the mechanical properties of forearm and vulvar skin were studied in 22 healthy women, 12 before and 10 after the menopause. The ratio between viscous deformation (Uv) and elastic deformation (Ue) and the biological elasticity, i.e. the ratio between immediate recovery (Ur) and total deformation (Uf), were both significantly lower in vulvar than in forearm skin. Ur/Uf decreased significantly with load in vulvar, but not in forearm skin, whereas Uv/Ue was not load-dependent in either site. Uv/Ue remained constant with age in both test sites, whereas Ur/Uf was significantly lower in post-menopausal women in both forearm and vulvar skin. In vulvar, but not in forearm skin, Uv/Ue was significantly correlated with body height which may be an indicator of mechanical connective tissue properties. Viscous deformation plays a lesser role and biological elasticity is decreased in vulvar compared to forearm skin. Despite differences in mechanical parameters at both sites, age-related changes seem to be similar.

During the past two decades, much progress has been made in the understanding of mechanical properties of the skin. The influence of physiological variables such as age, sex and skin thickness on the rheological properties of skin have been studied, and disease- and drug-induced changes in the biomechanics of skin have been characterized.[1] The mechanical properties of genital and especially vulvar skin, however, have not attracted interest, which may be partly due to problems of instrumentation. This skin is subject to considerable mechanical stress in sexual activity and in childbirth, and vulvar skin provides a unique model for the study of age- and hormone-related changes without the interference of actinic damage.

As part of an ongoing project on the characterization of vulvar skin as a model for specialized skin, we have studied the mechanical properties of vulvar and forearm skin in pre- and post-menopausal women.

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METHODS

Standards for determination of mechanical properties of skin

In an attempt to define international standards for measuring physical properties of skin, a list of variables to be controlled or measured in *in-vivo* experiments was proposed at the Cardiff Symposium on Bioengineering and the Skin in 1979.[2] The variables include instrument–skin interaction, skin thickness, demographic parameters of the study population and methods of data reduction. This report follows the above standards.

Study population

Twenty-two healthy female volunteers, 12 premenopausal and 10 post-menopausal, gave informed consent to enter the study, which had been approved by the UCSF Committee for Human Research. The premenopausal women were between 28 and 49 years of age (mean 34·1, SEM 1·6 years) and the post-menopausal women were between 55 and 78 years (63·3 ± 2·1 years). Before the study, body height and weight were measured and a gynaecological history taken.

Measurements

All measurements were performed after the subjects had been physically inactive for at least 15 min. Before measurements were made on vulvar skin, hairs were clipped off the labia majora. The measuring sites were the volar side of the forearm midway between the wrist and the elbow, and the centre of the labium majus. All experiments were performed under neutral environmental conditions (room temperature 19–23°C, relative humidity 45–55%).

Suction device

The mechanical properties of the skin were evaluated using a recently developed skin elasticity meter (Cutometer SEM 474, Courage and Khazaka, Cologne, F.R.G.), which is based on the suction method. A vacuum is applied perpendicularly to the skin surface and the resulting skin deformation is measured. Since the test area is only about 3 mm^2, the contribution of the dermo-subepidermal interface to the deformation, a drawback with previous, much larger suction devices, is negligible. The small size of the test area is essential for studies in vulvar skin, which is not uniform in its mechanical characteristics. The main unit of the meter contains a pump generating a vacuum of up to 500 mbar, the pressure sensor and the electronical circuit controlling the pump and the analog/digital data conversion. A hand-held probe is attached to the main unit with two rubber tubes and the suction head is centred in the probe shield. It has a 2-mm circular opening for application of the vacuum to the skin and is fitted to a spring, which ensures that the head is applied to the skin with constant pressure. The skin deformation is measured with an accuracy of 0·01 mm and at a frequency of 100 Hz.

The Cutometer must be linked to an IBM PC/AT or compatible computer through the RS 232 serial interface, and it is completely controlled from the computer. A control program is provided by the manufacturer. Two measuring modes are available, a stress/strain mode and a time/strain mode. In the stress/strain mode, the vacuum is increased from 0 to 500 mbar over a chosen period of time, and the deformation (in mm) is displayed as a function of negative pressure (in mbar). In the time/strain mode, a chosen vacuum is applied for a chosen time period and the deformation is displayed as a function of time.

In this study, the time/strain mode was used. The vacuum (= load) was applied to the skin for a period of 5 s followed by a 3 s relaxation time. The skin deformations in response to the second

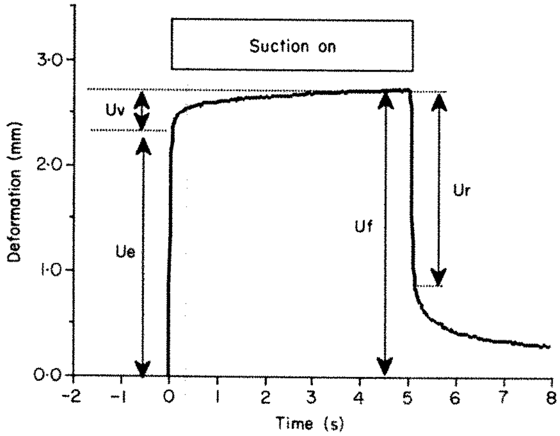

FIGURE 1. Typical registration of an experiment with the Cutometer on vulvar skin. A load (vacuum) of 500 mbar was applied for 5 s followed by a 3 s relaxation period. The parameters used to describe the deformation are as follows: Ue is the instantaneous deviation length, Uv is the viscoelastic and viscous deviation length (creep), Uf is the final deviation length and Ur is the instantaneous recovery length.

and each following suction period are similar, but are different from the first deformation, which is considered to be the preconditioning phase,[3] and was not evaluated. Recordings were made for three vacuum settings, 100, 300 and 500 mbar and the recording of a typical experiment is shown in Figure 1. The deformation of the skin during a loading cycle resembles that observed with other methods.[4–7] The deformation curve is composed of a fast deformation representing a purely elastic part, followed by a viscoelastic and finally purely viscous part. The same elements can be differentiated in the retraction of the tissue.

The nomenclature proposed by Agache *et al.*[4] was followed which defined Ue as the immediate deformation or skin extensibility, Uv as the delayed distension, Uf as the final deformation, and Ur as the immediate retraction. The Cutometer program offers a cursor mode for the measurement of these parameters directly from the graphical display. However, in order to reduce investigator bias and to automate data reduction, we transferred the acquired raw data to a spreadsheet (Lotus 1-2-3) and extracted the parameters in a standardized manner for all experiments. The values at 90 ms after application and removal of the load were taken as the immediate reaction values (Ue and Ur).

Presentation of results

All of the parameters Ue, Uv, Uf and Ur are a function of skin thickness and the results should be standardized for skin thickness.[1,8] In a preliminary study, skin thickness measurements were performed in forearm and vulvar skin with an A-scan ultrasound (Sonometrics DBR 300, Ocuserv, West Orange, NJ, U.S.A.) in a modification of the method described by Alexander and Miller.[9] In contrast to forearm skin, inter-assay and inter-investigator variance of skin thickness measurements was unacceptably high for vulvar skin, due to the fact that the echo of the dermo–subdermal interface was more difficult to identify at the vulva. This finding reflects the histological observation that in vulvar skin the deep dermal boundary as determined by the diameter of blood vessels is far from distinct.[10]

This problem was overcome by analysing ratios of the above parameters instead of the parameters themselves. Certain ratios of these parameters are biologically meaningful, do not depend on skin thickness and can be compared between sites and subjects.[5,8,11] The following ratios were computed: Uv/Ue is the ratio between delayed and immediate deformation and indicates the relative contributions of the viscoelastic plus viscous and the elastic deformation to the total deformation. Ur/Uf, the ratio of immediate retraction to the total deformation, is called biological elasticity.[8] Since it was found that the ratio Ur/Ue[4] closely paralleled Ur/Uf in our study, results of Ur/Ue are not presented.

Statistical methods

Statistical computations were performed with a statistical package (SPSS-PC+™, SPSS, Chicago, IL, U.S.A.) on an IBM XT-compatible computer. Differences between means were checked for significance using Student's *t*-test for paired data (procedure T-TEST). The influence of the independent factors load, menopausal status, height, weight, and number of births on the dependent variables Uv/Ue and Ur/Uf was evaluated using analysis of variance (procedure ANOVA).

RESULTS

Uv/Ue, the ratio between the viscous and the elastic deformation of the skin, was approximately 0·5 in forearm skin (Table 1), and was significantly lower in vulvar skin (approximately 0·2) for all load settings. The age-dependence of Uv/Ue is shown in Figure 2 with no significant differences between the age groups. This was confirmed by the results of the variance analysis. However, there was a significant positive correlation between the height of the women and vulvar Uv/Ue, with the taller women having higher Uv/Ue values, whereas none of the other independent variables tested influenced Uv/Ue significantly in vulvar or forearm skin (Table 2).

Ur/Uf, the biological elasticity, was significantly lower in vulvar than in forearm skin for all loads (Table 1). Whereas forearm Ur/Uf showed no variation with the pressure setting, vulvar Ur/Uf was negatively correlated with the load, i.e. the higher the deformation, the smaller the proportion of the immediate recovery. Figure 3 shows the influence of age on Ur/Uf, which seems to be of the same magnitude for vulvar and forearm skin and does not change with the load: the Ur/Uf ratio in post-menopausal women is 0·15–0·20 lower than in premenopausal women. The variance analysis confirms this, showing that menopausal status is a factor

TABLE 1. Mechanical parameters of forearm and vulvar skin ($n = 22$). Load is the vacuum applied to the skin. Results are given as mean ± SEM. P = probability of error in the *t*-test

	Load (mbar)	Forearm	Vulva	P
Uv/Ue	100	0·47 ± 0·07	0·25 ± 0·02	< 0·01
Uv/Ue	300	0·45 ± 0·04	0·21 ± 0·02	< 0·001
Uv/Ue	500	0·51 ± 0·04	0·22 ± 0·02	< 0·001
Ur/Uf	100	0·76 ± 0·03	0·67 ± 0·03	< 0·05
Ur/Uf	300	0·77 ± 0·03	0·57 ± 0·04	< 0·001
Ur/Uf	500	0·78 ± 0·03	0·47 ± 0·04	< 0·001

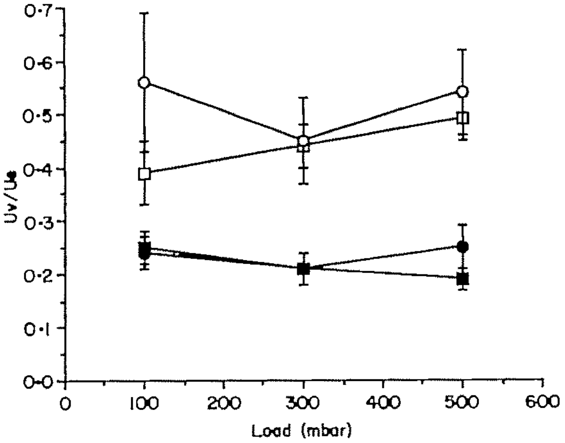

FIGURE 2. Influence of load (vacuum applied to the skin) on Uv/Ue in forearm and vulvar skin of pre- and post-menopausal women. Uv/Ue is higher at the forearm than at the vulva in both pre- and post-menopausal women, but load does not influence Uv/Ue significantly at either site. Age-related differences are not apparent. —□—, forearm, premenopausal; —■—, vulva, premenopausal; —○—, forearm, post-menopausal; —●—, vulva, post-menopausal.

TABLE 2. Variance analysis (ANOVA) of Uv/Ue in forearm and vulvar skin ($n = 22$). The results of the variance analysis with Uv/Ue as the dependent variable and load, menopausal status, height, weight and births as the independent variables. The test for main effects evaluates whether any of the independent variables influences the dependent variable significantly. If so, the percentage of variance of the dependent variable explained by the independent variable and the significance of a contribution of that variable to the total variance is computed. P = probability of error in the F-test; NS, not significant; NA = not applicable, since test for main effects not significant

	Forearm*	Vulva†	
Variable	% variance explained	% variance explained	*P*
Load	NA	3	NS
Menopause	NA	0	NS
Height	NA	27	<0·001
Weight	NA	<1	NS
Births	NA	3	NS

Test for main effects * *P*, NS; † *P*, <0·001.

influencing Ur/Uf significantly in both the forearm and the vulva, and that in vulvar, but not in forearm skin, the load is a second significant factor (Table 3).

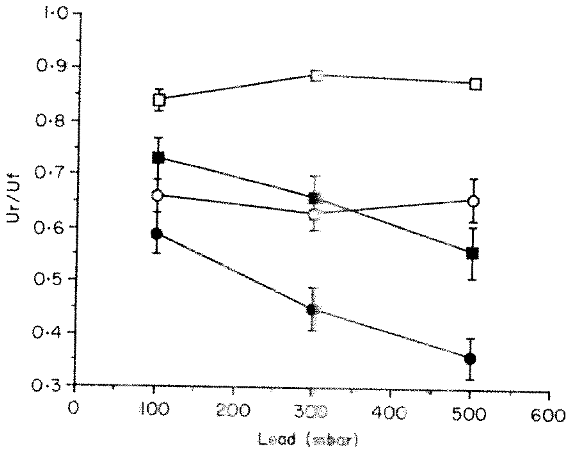

FIGURE 3. Influence of load (vacuum applied to the skin) on Ur/Uf in forearm and vulvar skin of pre- and post-menopausal women. Ur/Uf is higher at the forearm than at the vulva in both pre- and in post-menopausal women. Ur/Uf decreases with the increase in load in vulvar, but not in forearm skin. Key as for Figure 2.

TABLE 3. Variance analysis (ANOVA) of Ur/Uf in forearm and vulvar skin ($n = 22$). The results of the variance analysis with Ur/Uf as the dependent variable and load, menopausal status, height, weight and births as the independent variables. The test for main effects evaluates whether any of the independent variables influences the dependent variable significantly. If so, the percentage of variance of the dependent variable explained by the independent variable and the significance of a contribution of that variable to the total variance is computed. P = probability of error in the F-test, NS, not significant

	Forearm*		Vulva†	
Variable	% variance explained	P	% variance explained	P
Load	0	NS	22	$< 0·001$
Menopause	50	$< 0·001$	21	$< 0·0001$
Height	0	NS	< 1	NS
Weight	2	NS	4	NS
Births	3	NS	< 1	NS

Test for main effect * P, $< 0·001$; † P, $< 0·001$.

DISCUSSION

Numerous methods have been developed for the evaluation of mechanical properties of the skin, including tensile, torsional, elevation, indentation, suction and vibration tests, which makes it difficult to compare results.[1] However, there is general agreement that the mechanical properties of normal skin depend on skin thickness, age and sex, and skin thickness is a function of body region, age and sex. This study quantitatively describes the significant differences in mechanical behaviour between forearm and vulvar skin for the first time.

Viscoelastic and viscous deformation (Uv) contributed far less to the total deformation (Uf) in vulvar compared to forearm skin. Since the viscous part of the deformation is attributed to the displacement through the fibrous network of the interstitial fluid containing highly viscous glucosaminoglycans,[12] and the elastic part is linked to the stretching of collagen and elastic fibres, the lower Uv/Ue of vulvar skin indicates that the biochemical composition of the dermis at the vulva is different from that at the forearm. Body height was found to be significantly correlated with Uv/Ue of vulvar skin, and more attention should be paid to this parameter which may be an indicator of mechanical connective tissue properties.

The biological elasticity Ur/Uf of vulvar skin is significantly lower than that of forearm skin, especially at higher loads. Since this parameter is correlated with the function of the elastic fibre network, elastic fibres are either less abundant or functionally different in the vulva. Vulvar skin appears to be an area of 'pre-aged skin'; its biological elasticity in younger women resembling the forearm skin of older women. The significant decrease of Ur/Uf with load probably reflects a higher extensibility of vulvar skin, which is not equalled by elastic fibre network retraction.

The results of the many studies on changes in mechanical properties of the skin during the process of aging are confusing and sometimes contradictory. For loads parallel to the skin surface, however, evidence is growing that the immediate distension Ue, when standardized for skin thickness, remains fairly constant in adulthood, until it decreases in the eighth decade indicating a higher skin stiffness.[11] The ratio between the viscous part Uv and the elastic part Ue had been reported to rise progressively with age,[11] but this finding was not reproduced in a recent study by the same group (Escoffier *et al.*, unpublished data). The results obtained here with a load perpendicular to the skin surface do not indicate age-dependent changes of Uv/Ue, but our post-menopausal group with a mean age of 63·3 ± 2·1 years may have been too young to detect these changes. The biological elasticity Ur/Uf decreases with age,[11] which was confirmed by our observations, and age-related Ur/Uf changes in vulvar skin do not seem to differ from those in forearm skin. The age-dependent decrease of Ur/Uf is explained by marked changes in the elastic fibres, such as increased porosity and disintegration, and of the elastic fibre network.[13,14]

Since hormone receptors are abundant in the vulva, it may be that, in addition to age, sex hormone levels modulate mechanical parameters of vulvar skin. A recent study on forearm skin in premenopausal women reported variations in the extensibility during the menstrual cycle.[15] Our grouping of volunteers into pre- and post-menopausal women combines the factors of age and sex hormone levels. However, since differences in Ur/Uf in vulvar and forearm skin of pre- and post-menopausal women parallel each other, hormone-related changes, which would be more visible in the vulva, do not seem to occur for Ur/Uf.

It is remarkable that childbirth, although a major mechanical trauma to vulvar skin, does not seem to be a variable influencing Uv/Ue or Ur/Uf, which may reflect the presence of an effective repair mechanism. The changes of mechanical properties in vulvar skin during and after pregnancy and delivery might provide a model for the study of connective tissue damage and repair.

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Reduced vasoactive responses in port wine stains

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SUMMARY

Changes in skin blood flow and haemoglobin content of port wine stains and clinically normal contralateral skin were determined by laser-Doppler flowmetry and reflectance spectrophotometry in 14 patients after application of a vasodilating cream containing methyl nicotinate 1·0%, glycol salicylate 10·0% and capsaicin BPC 0·1% (Algipan cream) and in 16 patients after application of a corticosteroid tape containing flurandrenolone (Haelan tape). Changes in blood flow measured after application of vasodilator cream were significantly reduced in port wine stains compared with contralateral skin ($P < 0{\cdot}01$). After application of the vasoconstrictor steroid tape changes in blood flow were significantly reduced in the port wine stains ($P < 0{\cdot}05$) and changes in haemoglobin content were also significantly reduced in the port wine stains compared with normal skin ($P < 0{\cdot}05$). These results support the concept of reduced autonomic innervation of cutaneous vasculature in port wine stains.

The development of laser therapy as a successful treatment of port wine stains, has led to renewed interest in these vascular naevi which affect 0·3–1% of the population.[1,2] The ectatic dermal blood vessels characteristic of port wine stains have an increased erythrocytic content which correlates with the clinical appearance of the naevus.[3] This ectasia is progressive with age and results in change in colour of the naevus from pink to purple. The ectatic dilatation occurs in the most superficial 0·8 mm of the dermis and involves mature dermal blood vessels that stain normally with antibodies directed against vessel walls.[4] The naevi often follow the distribution of sensory nerves, most commonly the trigeminal. Smoller and Rosen[5] have demonstrated a reduction in perivascular nerves in port wine stains which led to the suggestion that port wine stains are due to an abnormal neural regulation of blood flow. This defect is likely to be autonomic in nature as there is no sensory loss over the naevi. This impaired neural regulation has been confirmed in two patients with port wine stains of the limbs.[6]

In a previous study, we demonstrated the value of laser-Doppler flowmetry and reflectance

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spectrophotometry as objective measurements of change in port wine stains.[7] We were also able to demonstrate that the normal skin in patients with port wine stains responds in a similar manner to that of controls. In this study we investigated the vascular reactivity of port wine stains to the percutaneous application of vasoactive substances and compared them with the normal contralateral skin.

METHODS

Patients

The responses to the application of a vasodilator cream were assessed in 14 patients with a port wine stain, and the responses to the application of a vasoconstrictor tape was assessed in 16 patients. This group included three patients who had been assessed after application of the cream on a separate occasion. The details of the patients are shown in Table 1. None of the patients was currently taking medication, nor had they smoked, eaten or had a drink for 2 h prior to measurements.

Measurements

All subjects were equilibrated in the laboratory for 30 min prior to measurements. Changes in skin colour were assessed by observation, photography and reflectance spectrophotometry in all patients. The latter provides a reproducible objective measurement of the total haemoglobin content of skin and its contribution to skin colour in the area assessed.[8,9] Cutaneous blood flow was measured using laser-Doppler flowmetry (Perimed, Stockholm, Sweden) in 17 patients. This instrument provides a reproducible and objective measurement of blood flow as a function of number and speed of movement of red blood cells in the area assessed, measured in mV.[10,11] It is impossible to ascertain whether changes in flowmetry are due to changes in absolute number of red blood cells or absolute changes in their flow rate. However, as reflectance spectrophotometry measures total haemoglobin content of skin, it may be inferred that changes in this index reflect changes in red cell number.

Vasodilator responses

The vasodilator cream used was the topical rubrifacient, Algipan®, containing methyl nicotinate 1·0%, glycol salicylate 10·0% and capsaicin BPC 0·1%. It produces a prompt erythematous reaction based partly on a direct effect on the cutaneous vasculature, and also via cutaneous nerves.[12]

TABLE 1. Patient details ($n = 27$, three patients are in both groups)

	Vasodilator group ($n = 14$)		Vasoconstrictor group ($n = 16$)	
Site	Male ($n = 6$)	Female ($n = 8$)	Male ($n = 5$)	Female ($n = 11$)
Face	4	6	5	11
Arm	1	—	—	—
Chest	1	—	—	—
Leg	—	1	—	—
Back	—	1	—	—

A circle 9·6 cm^2 in area was marked out in the centre of the port wine stain and on the contralateral skin using the plastic template for the laser-Doppler probe. Baseline readings were taken from the centre of this area. 0·2 cm^3 of the Algipan cream was rubbed uniformly into the two areas and removed after 3 min by gentle wiping. Measurements were taken from 5 to 10 min after initial application of the cream and the maxima recorded. Readings immediately after application of the cream were avoided since mechanical stimulation of the cutaneous circulation from rubbing may produce a prompt increase in blood flow as measured by laser-Doppler flowmetry, which falls after 5–10 min (S.Lanigan and P. Dowd, unpublished observations).

Vasoconstrictor responses

Flurandrenolone tape (Haelan tape®) provides a quantifiable dose of corticosteroid (4 μg/ml^2) to the area assessed and enables assessment of vasoconstriction in the area. Corticosteroids produce vasoconstriction by enhancing vascular reactivity to locally released noradrenaline.[13,14]

Patients with port wine stains attending for laser therapy were sent pre-cut strips of flurandrenolone tape measuring 2·5 × 3·5 cm, with a letter instructing them to apply the tape to an untreated area of port wine stain and to the contralateral normal skin 12 h before their appointment time. At their appointment and before removal of the tape, measurements were made as above on adjacent untreated port wine stain and contralateral normal skin. If the tape was inappropriately placed so that adjacent measurements were impossible these patients were not included in the study. Measurements were repeated in the areas under the tape 15 min after the tape had been removed.

Statistical analysis

Statistical analysis was performed by an analysis of variance and Newman–Keuls tests on logged data when comparing means of groups, and the Wilcoxon's rank sum test applied to paired data.

RESULTS

Visual changes

It was possible to observe pallor at sites of tape application, especially in normal skin, and erythema at sites of Algipan application. It was difficult to assess erythema in many port wine stains because of background colour. The changes observed are shown in Table 2.

Vasodilator responses

Measurements of reflectance spectrophotometry and laser-Doppler flowmetry in normal and port wine stain skin before and after application of the vasodilator cream are shown in Table 3. Both indices were significantly elevated in port wine stains with or without vasodilator when compared with normal skin. This difference was lost after application of the cream to normal skin and the mean blood flow exceeded that of the port wine stain. In addition, there was a significant increase in mean blood flow after application of vasodilator cream to normal skin, but not in port wine stains.

The percentage change in each variable measured at each site was calculated, with an increase in redness or blood flow after application of the cream as a positive result (Fig. 1). The response to vasodilator was reduced in port wine stains compared to normal skin. The percentage increase in blood flow was significantly greater in normal skin compared to contralateral port wine stains ($P = 0{\cdot}01$). The percentage change in blood flow (all figures positive) was also significantly

TABLE 2. Observed changes

Group/effect	Number
Vasodilator ($n = 14$)	
No effect	2
Equal redness	0
PWS* more red	0
Normal skin more red	2
Normal skin red, PWS no change	10
Vasoconstrictor ($n = 13$)	
No effect	1
Equal pallor	1
PWS paler	1
Normal skin paler	4
Normal skin paled, PWS no change	6

* PWS, port wine stain

TABLE 3. Vasodilator group measurements

Group	Mean (95% confidence limits)
Reflectance spectrophotometry ($n = 14$)*	Absolute units
1 Normal skin	126·5 (91·2–161·8)
2 Normal skin + vasodilator	158·2 (117·7–198·7)
3 Port wine stain	203·7 (167·7–239·7)
4 Port wine stain + vasodilator	220·3 (198·2–252·4)
Laser-Doppler flowmetry ($n = 11$)†	mV
1 Normal skin	22·2 (14·1–30·3)
2 Normal skin + vasodilator	66·3 (40·1–92·5)
3 Port wine stain	39·3 (29·5–49·1)
4 Port wine stain + vasodilator	56·7 (43·0–70·4)

* Significant differences ($P < 0·05$): 1 vs 3, 1 vs 4.
† Significant differences ($P < 0·05$): 1 vs 2, 1 vs 3, 1 vs 4.

greater in normal skin compared to port wine stain ($P < 0·01$). Whilst the difference was not significant ($P < 0·052$), the increase in haemoglobin content measured by reflectance spectrophotometry tended to be smaller in the port wine stain when compared with normal skin. There was no difference in the percentage change in haemoglobin content (all figures positive) between normal skin and port wine stains because of the large reduction in one port wine stain. It can be seen that a reduction in haemoglobin content and blood flow after application of vasodilator occurred in two port wine stains.

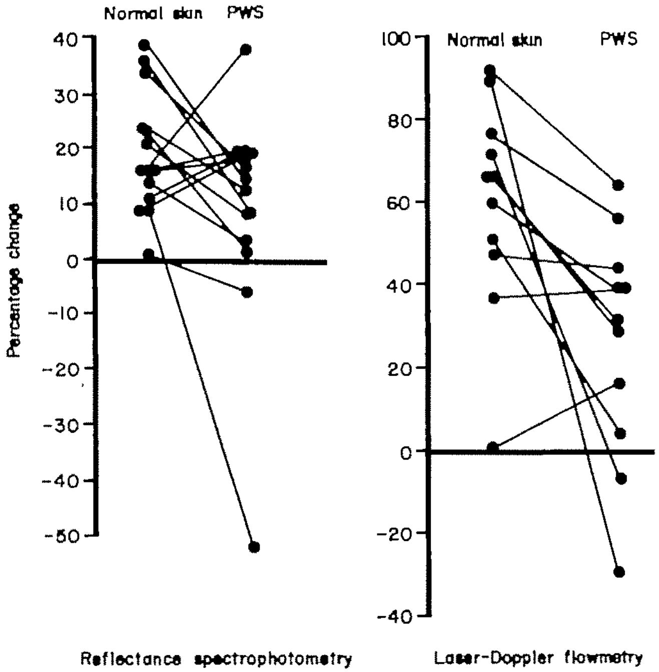

FIGURE 1. Vasodilator studies. Percentage increase in normal skin and port wine stains (PWS).

TABLE 4. Vasoconstrictor group movements

Group	Mean (95% confidence limits)
Reflectance spectrophotometry ($n = 16$)*	Absolute units
1 Normal skin	117·5 (79·3–155·7)
2 Normal skin + vasoconstrictor	97·3 (57·2–137·4)
3 Port wine stain	213·8 (158·2–269·4)
4 Port wine stain + vasoconstrictor	194·5 (156·3–232·7)
Laser-Doppler flowmetry ($n = 6$)†	mV
1 Normal skin	25·3 (10·3–40·3)
2 Normal skin + vasconstrictor	23·2 (13·8–32·6)
3 Port wine stain	61·6 (11·9–111·3)
4 Port wine stain + vasoconstrictor	61·5 (16·7–106·3)

* Significant differences ($P < 0{\cdot}05$): 1 vs 3, 1 vs 4, 2 vs 3, 2 vs 4.

† Analysis of variance: $F = 3{\cdot}27$ ($P = 0{\cdot}042$), no significant differences.

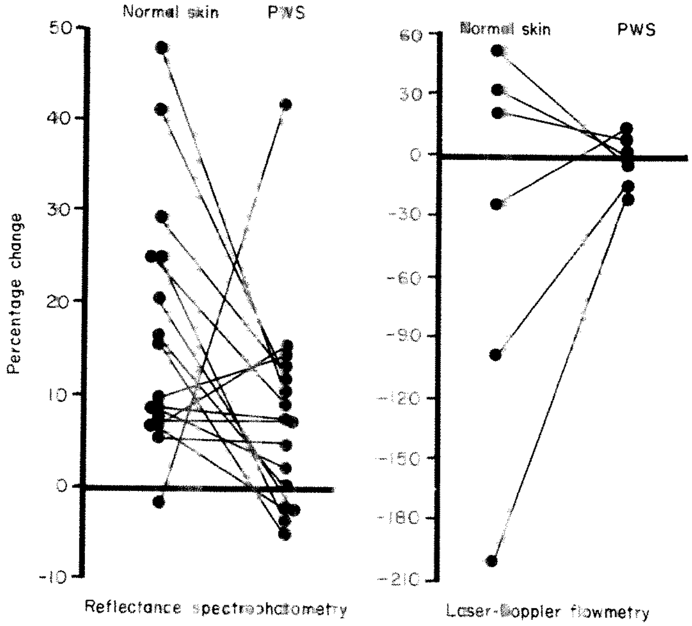

FIGURE 2. Vasoconstrictor studies. Percentage reduction in normal skin and port wine stains (PWS).

Vasoconstrictor responses

Measurements of skin colour and blood flow in port wine stains and normal skin in areas with and without tape are shown in Table 4. Application of Haelan tape resulted in reduced haemoglobin content of the normal skin and of the port wine stain which was not significant. There were no significant changes in skin blood flow as measured by laser-Doppler flowmetry.

The percentage reduction in blood flow and haemoglobin content induced by the tape was calculated at each site, with a decrease in flow or reflectance as a positive result (Fig. 2). There was a significantly greater percentage reduction in haemoglobin content as measured by reflectance spectrophotometry in normal skin compared with port wine stains ($P < 0{\cdot}05$). This difference was sustained when comparing percentage change (all figures positive) ($P < 0{\cdot}05$). There was no significant difference in the reduction in blood flow in normal skin and port wine stains. It can be seen that in three patients there was a large increase in blood flow at the site of tape application and the percentage change in blood flow (all figures positive) was significantly greater in normal skin compared with contralateral port wine stains ($P < 0{\cdot}05$).

DISCUSSION

It is of value to be able to measure changes in port wine stains objectively both in assessing responses to therapy and providing further information on the pathogenesis of these naevi. Non-invasive methods have included photography, thermography,[15] transcutaneous microscopy,[16] reflectance spectrophotometry[17] and laser-Doppler flowmetry.[18] Reflectance spectrophotometry and laser-Doppler flowmetry were used in this study as they provide reliable and reproducible measurement of skin colour and cutaneous blood flow.

The aims of this study were to investigate the vascular responsiveness of port wine stains and contralateral normal skin to a known vasodilator and vasoconstrictor. If the neural modulation of port wine stains is reduced it was hypothesized that the responses to vasoactive agents would be quantitatively different within these naevi. Our results show a statistically significant reduction in the response of port wine stains to vasodilator and vasoconstrictor stimuli when compared with contralateral clinically normal skin.

As the stimuli were applied percutaneously it is possible that the impaired responses in port wine stains were due to reduced absorption of the drugs. However, as the only histological differences between normal skin and port wine stains are the ectatic blood vessels in the dermis, this possibility is unlikely. A further explanation of these results is that the absorbed vasoactive agents are removed from the area more rapidly by the increased circulation in the naevus. This is unlikely, as the time to maximal vasodilation and resolution induced by Algipan was similar in port wine stains and normal skin. Because port wine stains and normal skin have different vascular anatomies, they will have different optical properties of the dermal layer which may result in altered penetration of the light into tissue. However, as haemoglobin is the major chromophore for both optical instruments, interaction with the incident light beams should occur at similar tissue depths. It may be that significant vascular changes are occurring deep within a port wine stain that are not measured by the instruments. It would remain the case, however, that the responses of the superficial vasculature are impaired.

Another explanation for the results would be a reduction in noradrenaline receptors on the vessel walls of port wine stains. This would explain both the ectatic nature of the vessels and also the impaired vasoconstriction, but not an impaired vasodilatory response. Finally, and most likely, these results are consistent with an impaired neural control of vascular reactivity within port wine stains.[5] Both vasodilatory and vasoconstrictive reactions were reduced, but not absent, suggesting a general reduction in autonomic control.

Cutaneous blood flow is under neural control with predominantly a vasoconstrictor tone at rest[13] with an important vasodilator system, particularly of the face, which causes increased blood flow in heat regulation.[14] It is of interest that patients with trigeminal ganglion ablation have a marked cutaneous flush. In some patients, the response to Algipan cream presented an appearance in their normal skin indistinguishable from their port wine stain. It was not possible, however, by vasoconstrictor application to alter the appearance of the port wine stain to that of normal skin. It is possible to completely blanch a port wine stain by intralesional injection of noradrenaline, but the threshold is higher than contralateral normal skin (unpublished observations). It is possible to both increase and decrease blood flow in port wine stains, so the ectatic vessels are unlikely to be fully dilated.

In conclusion, we have demonstrated an impaired vasoactive response in port wine stains to both vasodilating and vasoconstricting stimuli. This supports the theory of a reduced neural control in the pathogenesis of port wine stains. In addition, these results suggest that the pharmacological modulation of port wine stains to improve their appearance may prove difficult.

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Contact dermatitis with negative patch tests: the additive effect of allergens in combination

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SUMMARY

We deduced on theoretical grounds that conventional patch testing would be inadequate for the detection of sensitivity to multiple allergens. Fourteen patients with positive patch tests to two unrelated allergens were studied and the response to those two allergens was measured when tested singly or in combination, using 10 different pair combinations from 15 common allergens. With serial dilutions in chloroform (14 patients) and paraffin (four patients), the response was related to the log–dose of the allergen, and change in skin-fold thickness corresponded well with clinical grading. Single allergens diluted below the threshold for a patch-test response gave a response when given in combination, the threshold for a response to one allergen being lowered by the presence of another. On the linear part of the dose–response curves the response to the mixture of allergens was additive, the combined response being the sum of the individual components. Approaching the plateau region the response to the combination was greater than to the individual allergens but less than the sum of the single responses. The same results were obtained with allergens in paraffin. We conclude that conventional single allergen patch testing by itself is inadequate for the diagnosis of contact dermatitis.

This study arose from the discovery, on purely theoretical grounds, of a major defect in methods of testing for contact allergy. Since it has been shown that elicitation of a patch-test response is log–dose related[1,2] it follows that some sensitized subjects will have a negative patch test, the frequency of such an occurrence depending upon the balance of sensitivity and the eliciting concentrations.[2] It follows that patients who are sensitized to more than one antigen, but to a degree insufficient to produce a positive patch test to one or more of them alone, would react to a combination of antigens by the simple summation of their independent sensitivity responses. Since it had already been shown that multiple allergies are common, their occurrence corresponding to the variation in degree of susceptibility to sensitization within a population,[2] it was concluded that existing methods of patch testing with single allergens would prove inadequate for the detection of sensitivity in patients with multiple sensitivities. We believe that this explains why negative responses occur with patch testing where there is a high index of clinical suspicion and when the patients themselves associate the disorder with a particular application[3] and also when the dermatitis may indeed improve with the avoidance of the application.

The response to common allergens, applied singly and in combination, was measured using both the standard subjective methods of application and grading of response used for patch testing, and a more accurate application of allergen with an objectively measured response, the use of which we have already established.[1]

METHODS

Patients

We studied 14 patients who were known to have positive reactions by standard patch testing procedures to at least two unrelated allergens known not to cross react or have a common association. A total of 15 different allergens were used from the standard batteries of common allergens (nickel, cobalt, thiuram mix, neomycin, lanolin, fragrance mix, paraphenylenediamine, morpholinyl mercaptobenzothiazole, quinoline mix, Balsam of Peru, potassium dichromate, diethyl dithiocarbamatol zinc, azo yellow, soframycin, propylene glycol).

Allergens in solution

Fresh solutions were made up immediately before the application of test patches to the patients. Nickel sulphate was made up as a 20% solution in water and cobalt chloride as a 1% solution in water. With other chemicals 200 mg of allergen (as supplied by Trolab for routine patch testing) was dissolved in 0·8 ml of chloroform and mixed thoroughly. Six successive twofold dilutions were made and 20 μl of each dilution, alone or in addition to another allergen, was pipetted onto an A1-test (Imeco) and allowed to evaporate. The combinations used were as follows: nickel and thiuram mix; nickel and chromate; nickel and propylene glycol; lanolin and fragrance mix; chromate and diethyl dithiocarbamatol zinc; nickel and morpholinyl mercaptobenzothiazole; nickel and paraphenylenediamine; fragrance mix and quinoline mix; quinoline mix and Balsam of Peru; diethyl dithiocarbamatol zinc and azo yellow. The patches were applied to each inner arm using Scanpor adhesive tape so that the combination of allergens on one arm was in a symmetrical distribution to that of the single allergens on the other. The position of the different concentrations was varied to overcome possible site variation.

The patches were applied for 48 h and at 72 h the response was measured as change in skinfold thickness by calipers and assessed clinically using the standard scale.[3]

To exclude an irritant effect of the various allergens when applied together, six normal volunteers, who had neither a history of contact sensitization nor positive patch tests, had patches applied using the highest doses of allergens used in all the combinations studied in the sensitized patients.

Allergens in white soft paraffin

Six successive twofold dilutions of standard patch-test allergens (Trolab) were made in white soft paraffin. A 0·5-cm length of allergen in paraffin (approximately 11 mg) from a standard 5 cm^3 volume syringe was placed on a Finn chamber on Scanpor adhesive (Epitest Ltd) alone and in addition to a second allergen. The chambers were applied to the inner arms and the responses measured as before.

RESULTS

Allergens in solution

Correlation between objective and subjective assessment of response. The change in skin thickness corresponded well with the clinical grading (Fig. 1), and a direct relationship was found between the dose of the allergen and the size of the response it produced (Fig. 2).

The response to single and combined antigens. Three patients showed no response to any of the concentrations used.

(*a*) *Subthreshold doses.* In each of six patients who were sensitive to allergens when tested individually, the threshold concentration at which a response occurred was established both by standard visual assessment and by measurement of change in skin-fold thickness for both of the two allergens. Two further subthreshold dilutions were made for each of the allergens and when a subthreshold dose of each allergen was combined on one patch, a measurable response was produced, the magnitude of which depended upon the dilutions used (Fig. 3 and Fig. 2 at lower doses). The thresholds at which a response occurred to the combination of allergens was significantly lower ($P < 0{\cdot}01$, Kruskal–Wallis test) than the thresholds for a response to the individual allergens.

(*b*) *Linear part of dose–response curve.* In each of the 11 patients studied, 2–6 antigen concentrations were used, which produced a patch-test response to the individual allergens on the linear part of the dose–response curve. A greater response was elicited by the dilutions of allergens in combination than to either of the individual allergens. This is shown in the upper part of Figure 2. Figure 4 shows the responses to combinations of allergens and the sum of responses to individual allergens alone when the responses to the two allergens individually and in combination at a given dose were all on the linear part of the three dose–response curves. The

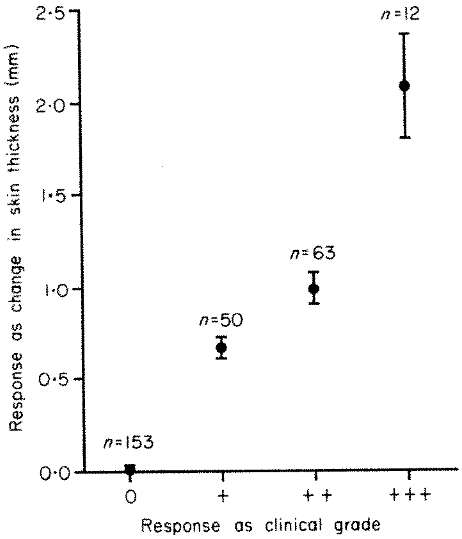

FIGURE 1. Response as change in skin thickness compared to clinical grade for antigens in solution. Results are shown for different allergen dilutions tested on 14 patients showing mean ± SEM of skin-fold thickness for each clinical grade.

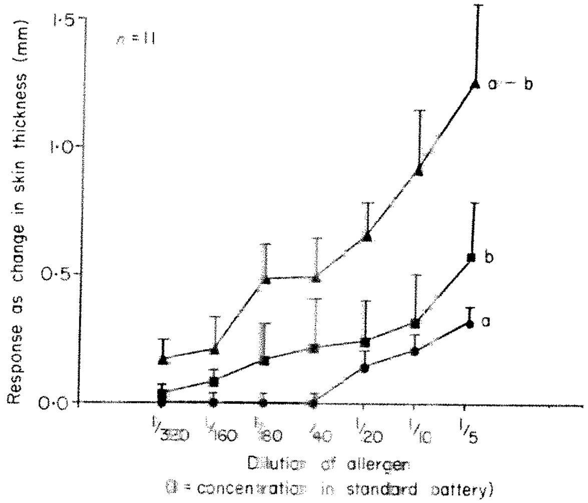

FIGURE 2. Response to dilutions of allergens in solution against log-dose of the allergen alone and as a mixture. Results are shown as mean ± SEM for 11 subjects. At each concentration a greater response is produced to the mixture than to either component allergen. a, first allergen; b, second allergen.

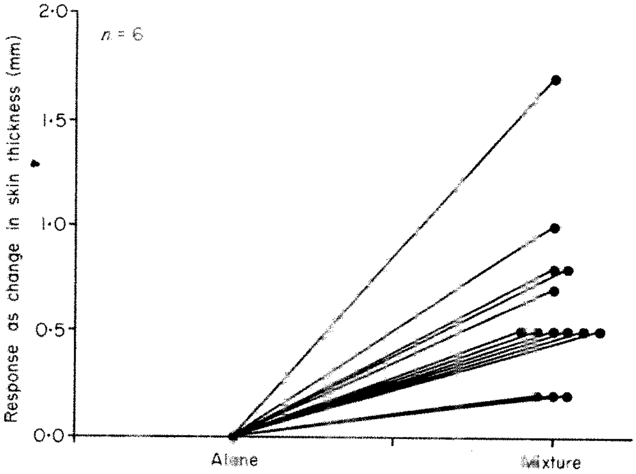

FIGURE 3. Response to mixture of allergens at subthreshold doses, i.e. when the response to both allergens individually was negative. Responses shown for six subjects.

slope of the curve is 45°, showing that the response to the mixture of the allergens is the same as the sum of the responses to the individual components. Thus the increase in response to the mixture of allergens was additive and not synergistic.

(*c*) *Plateau region of dose–response curve.* Since the concentrations of the allergens used were not increased sufficiently to ensure that the plateau of the dose–response curve had been reached, the highest concentrations can only be presumed to represent the upper part of the curve in the region of the plateau in the patients studied. The responses to the combination of allergens were significantly greater than the responses to the individual allergens alone

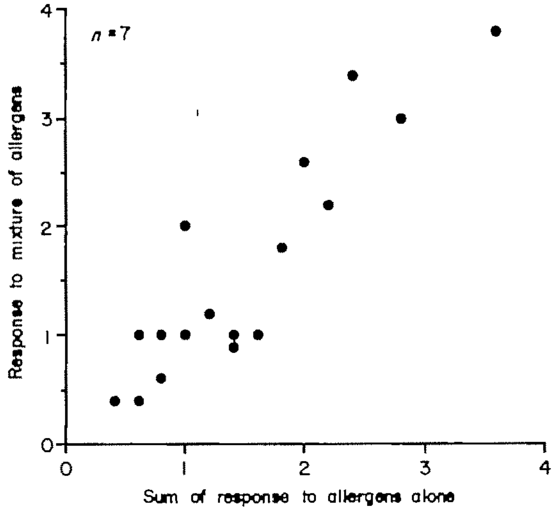

FIGURE 4. Comparison of response to mixture of two allergens with sum of their individual responses from seven subjects where responses to allergens alone and in mixture were on the linear parts of their log dose–response curves. The response is approximately the same to the mixture as to the sum of the individual responses.

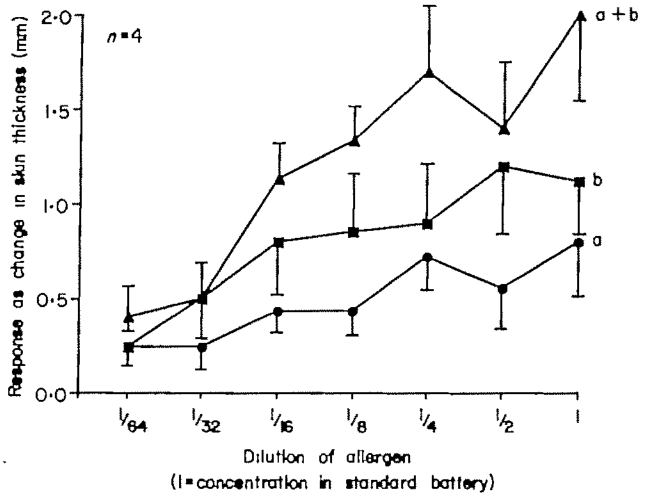

FIGURE 5. Response to allergens alone and as a mixture when diluted in paraffin in four subjects. The dose–response relationship and augmentation of response in the mixture were the same as found with the allergens in solution (Fig. 2). a, first allergen; b, second allergen.

($P<0{\cdot}01$, Kruskal–Wallis test) but were less than the arithmetical sum of the individual responses ($P<0{\cdot}01$ sign test).

(*d*) *Response to allergens in non-sensitized subjects.* In six normal subjects with negative patch tests, mixtures of all allergens studied were applied in the highest concentrations used. None produced a positive reaction.

Allergens in paraffin

To confirm that the findings were applicable to standard methods of patch testing, the allergens were diluted in white soft paraffin and applied to four patients. As with the allergens in solution we found a direct relationship between the dose of the allergen and the size of the response it produced (Fig. 5), and a greater response was elicited by the allergens in combination than to either of the individual allergens throughout the concentrations used in each of the four subjects. As with soluble allergens the response to the combination was additive, the sum of the response to the two allergens alone being no greater than that of the allergens in combination.

DISCUSSION

Patch testing with common contact dermatitis-producing allergens confirmed the expected log dose–response relationship. The responses to mixtures of antigens were, as predicted, the result of simple additive effects depending on their position on the discrete dose–response curves, and positive patch tests to combinations of allergens occurred at concentrations which gave negative reactions singly.

Although the classical sigmoid dose–response relationship was apparent for each of the substances studied, the study did not set out to define precisely the threshold, log–linear and plateau phases for each allergen because the main purpose was to establish working dilutions for the experiments with mixtures of allergens. Using dilutions derived in this way it was found that when allergens were tested in combination in patients with two known sensitivities, the response was invariably greater than to the single allergen. This was not an artefact of the application technique of dilutions of soluble allergens or the measurement of response by lesion thickness, because this method gives a dose–response relationship with the common allergens as well as with experimentally-induced sensitization.[1] In this study the responses as measured by skin thickness were shown to be a good objective measure of the subjective gradings. The enhanced mixed response occurred with all 15 allergens studied despite their totally unrelated chemical nature and widely differing dilutions, so enhanced passive diffusion of allergens can be excluded as a mechanism. An irritant effect of the combination of various allergens can also be excluded, because we found that a combination of the highest concentrations of all the chemicals used did not produce a response in patients who had no evidence of contact sensitivity to single allergens. It can therefore be concluded that the enhanced response of allergens applied together was due to a true increase in the magnitude of the contact dermatitis reaction. Since the enhanced response occurred with all the common but unrelated sensitizers studied, it can also be concluded that the observed augmentation is a general phenomenon and not allergen specific.

Having demonstrated the phenomenon of augmentation which had been predicted on theoretical grounds, the next set of studies was designed to define its mechanism. Two mechanisms were considered: synergy, the immune response to one allergen being synergistically augmented in the presence of one or more others, and addition by the simple additive sum of the inflammatory responses. To distinguish between the two possibilities combinations of allergen concentrations were used at different points on the dose–response curves. With a simple additive effect, if concentrations of one or more allergens below the individual response thresholds are applied together, they will produce a response greater than the sum of the individual responses whenever simple summation of effect passes the threshold of response. In other words, below the threshold a purely additive action will be indistinguishable from synergy. On the linear part of the dose–response curve the combined response would be additive provided that the sum of the responses falls short of the plateau. By contrast, a truly synergistic response would show a greater than additive effect both below the threshold and on the linear part of the curve. The response at the plateau region would not allow a distinction because of the limitation imposed by the skin itself on any further inflammation. A synergistic mechanism could therefore only be distinguished by this method on the linear phase of the curve.

The responses at different parts of the curve were studied and the findings conformed completely to the theoretical predictions of a biologically additive response. Thus, reactivity greater than the arithmetical sum of the individual responses was seen when subthreshold doses were used. This was due to subthreshold addition and not synergy because the responses were always additive when the total response remained on the linear part of the dose–response curve. Therefore, the augmentation of response found when two or more allergens were applied together was simply additive. Although the plateau was not studied in detail, the increase with the two allergens together was less than additive which would be expected because of limitation of further volume expansion. In the absence of synergy, therefore, it is not necessary to invoke any change in immune handling of the allergens or reactivity to them when more than one are applied simultaneously.

The quantitative studies upon which these conclusions are based used dilutions of soluble allergens and lesion thickness for measurement of response. The possibility that the results were an artefact of this technique could be excluded because the same summation of responses was found using allergens in conventional bases and using conventional patch testing procedures. Thus, these findings are relevant to clinical testing and have practical as well as theoretical consequences.

Contact allergy occurs in an environment where exposure to multiple antigens and multiple sensitivity is common. About 40% of our patients who have a positive result on testing with the standard European patch-test battery are positive to more than one of the allergens. Since elicitation of sensitivity is dose-responsive, and, as we have shown, additive, it follows that contact dermatitis may be precipitated by a specific allergen or allergens in individuals with minor or subclinical sensitivities in whom conventional patch tests to individual allergens are negative. It is remarkable that this possibility and its simple quantitative explanation have never been investigated despite the clinical observation of negative single patch-test responses in patients with apparent contact sensitivity.[4,5] Not only will the response to the various allergens summate to enhance the response but also, as has been shown, the *threshold* to one particular allergen is reduced in the presence of other allergens to which the patient is also sensitive. The lowering of threshold which we observed with mixtures of two allergens will be even more profound with the greater number of sensitivities often seen in practice. The concept of threshold is crucial to an understanding of relevance of a positive patch test, since it is not the simple presence or absence of a positive patch test but its magnitude as reflected in the threshold concentration which determines whether or not a patient will react to a chemical in a particular product.

It follows from our work that the use of single concentrations of single allergens is inadequate for the investigation of contact dermatitis. The simplest way to overcome this diagnostic defect is the use of mixtures of various allergens and of different concentrations, although further study will be required to optimize these. In summary, by dilution of common allergens to concentrations that do not themselves produce reactions, this study has shown that mixtures of the allergens applied together will produce immune contact patch-test responses. This has considerable theoretical significance for the understanding of dermatitis reactions as well as practical implications about the nature of patch testing.

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Skin-derived antileucoproteases (SKALPs): characterization of two new elastase inhibitors from psoriatic epidermis

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SUMMARY

Elastase inhibiting activity (EIA) was demonstrated in the epidermis from lesions and in psoriatic scale, whereas normal epidermis did not contain significant EIA. Two new elastase inhibitors were partially purified and characterized using psoriatic scale as a source. The two species (approximate molecular weights 10 and 20 kDa) were shown to be stable, and high-affinity inhibitors of human leucocyte elastase ($K_i < 10^{-10}$ M). No activity against human cathepsin G could be demonstrated. Cultured human keratinocytes were shown to contain EIA activity similar to that found in psoriatic scale. EIA could also be demonstrated in human epidermis following the induction of an experimental inflammatory response by sellotape-stripping. We propose the acronym SKALP (skin-derived antileucoprotease) as a name for these new proteinase inhibitors.

Proteinases derived from polymorphonuclear leucocytes (PMNs), such as elastase and cathepsin G, can cause tissue damage when secreted, since they are active at a neutral pH and use a broad spectrum of connective tissue molecules as substrates.[1] In order to control proteolysis by these enzymes, a number of highly effective proteinase inhibitors are present in the extracellular space.[2] These are either present systemically, such as α-1-proteinase inhibitor (α-1-PI), or they are locally synthesized such as anti-leucoprotease or secretory leucocyte protease inhibitor in the lungs[3] and parotid glands.[4] However, proteinases can escape the action of proteinase inhibitors and this results in extensive tissue damage. Oxidation of α-1-PI has been implicated in the development of lung emphysema,[5] and PMN-derived proteinases have been shown to lead to the breakdown of cartilage *in vitro*[6] and glomerular injury *in vivo*.[7] No definitive role for PMN-derived neutral proteinases has so far been demonstrated in skin disorders but PMN elastase and cathepsin G can degrade components of the epidermal basal lamina *in vitro*, thereby dissociating epidermis from the dermis.[8]

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In previous studies we have shown that human epidermis in various inflammatory skin disorders[9] or epidermis exposed to experimental inflammation[10] contains significant amounts of elastase-inhibiting activity (EIA), but this activity is not found in normal skin. We present the preliminary biochemical characterization of two elastase inhibitors which are tentatively named SKALPs (skin-derived antileucoproteases).

METHODS

Chemicals

Methoxysuccinyl-alanyl-alanyl-prolyl-valyl-7-amino-4-methylcoumarin, N-succinyl-phenyl-alanyl-leucyl-phenylalanyl-thiobenzylester and carbobenzoxy-L-arginyl-7-amino-4-methyl-coumarin were obtained from Bachem, Bubendorf, Switzerland. Cetyltrimethylammonium bromide (CTAB) was obtained from ICI, U.K. Affigel-10 and all reagents for sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Biorad Laboratories, Richmond, CA, U.S.A. Sephadex G-50 (fine grade) was from Pharmacia Fine Chemicals, Uppsala, Sweden. Dithio(bis)nitrobenzoic acid, α-1-proteinase inhibitor (α-1-PI), phenylmethylsulphonylfluoride, trypsin, porcine pancreatic elastase and phenylbutylamine were from Sigma Chemicals, St Louis, MO, U.S.A.

Active-site titrated elastase, used for calibration procedures, was a kind gift from Dr J.A.Kramps, Department of Pulmonology, University Hospital, Leiden, The Netherlands.

Skin samples

Biopsies were taken from patients with stable chronic plaque psoriasis and from normal healthy volunteers, using either a razor blade in conjunction with a metal guard, or a Castroviejo keratotome as previously described.[10] The biopsies were at a thickness of 0·3 mm for lesional skin, 0·2 mm for the normal skin and the uninvolved skin in the patients with psoriasis. They were washed in phosphate-buffered saline (PBS) and immediately processed for extraction. The scale from the skin of patients with psoriasis were stored at −20°C until use. Normal stratum corneum was obtained from the backs of healthy volunteers by gentle scraping with a scalpel blade, and stored at −20°C until further use. The skin of normal volunteers was repeatedly tape-stripped[10] and biopsied 48 h later. The scales and biopsies were processed to determine the elastase inhibitory activity as previously described.[10]

Leucocyte preparation

Human PMNs were purified from peripheral blood using standard techniques of dextran sedimentation and Ficoll gradient centrifugation, and extracted in buffer (100 mM Tris, 1 M NaCl, 0·3% CTAB, pH 8·5).[11]

Purification of leucocyte elastase and cathepsin G

Elastase and cathepsin G were purified from leucocytes obtained by lymphopheresis of a patient with chronic myeloid leukaemia. Briefly, myeloid cells were subjected to differential salt extraction (0·1 M NaCl to extract the bulk of elastase, and 1 M NaCl to extract the bulk of cathepsin G), and the enzymes were purified on a phenylbutyl-Affigel-10 affinity column.[12,13] Samples were dialysed against 1 M NaCl and stored at −20°C.

Assay of elastase and elastase-inhibiting activity

The elastase activity was measured according to methods previously described[11] using the fluorogenic substrate methoxysuccinyl-alanyl-alanyl-prolyl-valyl-aminomethylcoumarin.[14] Inhibition of elastase activity by skin samples was routinely measured as the percentage inhibition of a standard amount of elastase. A sensitive microassay[11] was used to enable measurement of small amounts of inhibitory activity. Briefly, 10 μl containing 1 ng purified elastase (or an extract of 500 PMNs which is equivalent to approximately 1 ng human leucocyte elastase) was mixed with inhibitor preparation (10 μl) and incubated at 37°C for 15 min to allow complexing of enzyme and inhibitor. Thereafter, 20 μl of substrate solution was added (250 μM in extraction buffer) and the reaction was allowed to proceed for 30 min at 37°C. The incubation was terminated by the addition of 1 ml sodium carbonate buffer (100 mM, pH 10·5) and fluorescence was measured at 375 nm (excitation) and 440 nm (emission). Inhibition of elastase activity in this assay was calculated as the percentage inhibition of the activity of 1 ng elastase (final concentration during the assay approximately 1 nM).

One unit of inhibitory activity was defined as the amount required to reduce the activity of 1 nM elastase by 50%. Elastase activity was linear during the assay (final substrate concentration 125 μM, of which less than 10% is converted during the 30-min incubation with 1 ng elastase). Inhibition of elastase activity by epidermal extracts was linear with the amount of inhibitor added, up to about 70% inhibition, in the assay as described (see Figure 4 for a typical inhibition curve).

Assay of cathepsin G and cathepsin G inhibition

Cathepsin G was measured colorimetrically using N-succinyl-phenylalanyl-leucyl-phenylalanylthiobenzylester as a coupling reagent to detect the free benzylthiol.[15] Inhibition of cathepsin G was measured by pre-incubating purified cathepsin G at 0·05–5 μg/ml (final concentration) with an excess of inhibitor preparations at 37°C, from 5 to 60 min. All the incubations were performed in 1 M NaCl, 50 mM sodium orthophosphate, pH 7·5. Thereafter, the cathepsin G activity was measured and compared to the reference sample that contained no inhibitor. Alpha-1-PI was taken as a positive control for inhibition of the enzyme.

Assay of trypsin and trypsin inhibitory activity

Bovine pancreatic trypsin was measured fluorimetrically using carbobenzoxy-L-Arg-aminomethylcoumarin as a substrate.[16] The assay was performed as described for leucocyte elastase, except for the buffer system, which consisted of a 50 mM Tris buffer pH 8·0 containing 10 mM $CaCl_2$ and 1% DMSO. Inhibition of trypsin was measured as the percentage inhibition of 50 ng trypsin under the given assay conditions.

Protein measurements

Protein in the eluent of Sephadex G-50 chromatography was monitored at 280 nm. For quantitative measurements a direct fluorimetric assay was used (278 nm excitation, 340 nm emission). The amount of protein was read from a calibration curve obtained with bovine serum albumin.

Gel permeation chromatography

One gram of psoriatic scale was homogenized in 20 ml PBS (yielding a preparation of about 10,000 to 15,000 units inhibitor/mg soluble protein). The extract was centrifuged and the clear supernatant was concentrated by vacuum evaporation and dialysed against PBS (final volume 1·5 ml). This sample was applied to a Sephadex G-50 (fine-grade) column (60 × 1·8 cm) equilibrated with PBS. The samples were eluted with PBS (flow rate 0·3 ml/min) and collected in 4-ml fractions. The fractions were measured for EIA as described above.

SDS-PAGE

Proteins were separated on a 16% polyacrylamide (5·4% crosslinker) slab gel according to Laemmli,[17] and visualized with Coomassie brilliant blue or silver staining. Proteins were recovered from the gel by extracting gel slices with distilled water for 24 h at 4°C. The extracts were concentrated by vacuum evaporation and tested for EIA.

Cell culture

Human epidermal or neonatal foreskin keratinocytes were cultured according to the Rheinwald and Green system.[18] Second passage keratinocytes were cultured to 60–90% confluence when used for extraction. The feeder cell layer (3T3-fibroblasts) was removed with EDTA and trypsin and the remaining keratinocytes were harvested either by trypsinization, or following scraping. The cells were washed extensively to remove traces of fetal calf serum and trypsin, and were extracted by sonication and homogenization. This preparation was used for the determination of elastase inhibitory activity in cultured keratinocytes. Human skin fibroblasts were cultured from human skin biopsies and following trypsinization the extraction was as described for keratinocytes. Similarly, human umbilical vein endothelial cells[19] (a kind gift of Dr R.M.W. de Waal, Department of Pathology, University Hospital, Nijmegen, The Netherlands), and human melanoma cells[20] (line IGR 39, a kind gift of Professor D.J.Ruiter, Department of Pathology, University Hospital, Nijmegen, The Netherlands) were harvested by trypsinization and extracted.

Statistics

Statistical evaluation was performed using the two-tailed Mann–Whitney *U*-test. *P* values smaller than 0·05 were considered to be statistically significant.

RESULTS

Elastase inhibiting activity in normal and psoriatic epidermis

The EIA of lesional and uninvolved psoriatic epidermis and normal epidermis was measured in extracts of biopsies. Tissue samples were homogenized in extraction buffer, containing the detergent CTAB. Table 1 shows that psoriatic lesional epidermis contained a more than sixtyfold higher concentration of EIA compared to that of normal epidermis. Uninvolved psoriatic epidermis contained hardly any inhibitory activity and was comparable to the normal epidermis. Inhibitory activity could be demonstrated regardless of the extraction procedure or the elastase assay (either in PBS or in buffer containing high salt and detergent). This was taken to indicate that inhibitory activity was not derived from traces of plasma in the samples, since the major plasma inhibitor of leucocyte elastase (α-1-PI) does not bind to elastase in the presence of the cationic detergent CTAB. In addition to the EIA in the biopsies, we measured EIA in the scale obtained from patients with psoriasis and normal stratum corneum. Table 1 shows that psoriatic scale contained over 400 times more inhibitor than normal stratum corneum. As with the epidermal biopsies, it was found that the scale extract could inhibit elastase even in the presence of a high amount of salt and detergent.

TABLE 1. Elastase inhibiting activity in normal and psoriatic epidermis

Tissue	EIA units/mg tissue (mean ± SD)
Normal epidermis	2·5 ± 1·4 (6)
Psoriatic uninvolved epidermis	1·3 ± 0·2 (3)
Psoriatic lesional epidermis	166 ± 249*(6)
Normal stratum corneum	2·6 ± 1·4 (6)
Psoriatic scales	1195 ± 708†(7)

Elastase-inhibiting activity was measured in extracts of normal and psoriatic epidermis. One unit of EIA was defined as the amount required to give 50% inhibition of 1 nM elastase in the standard assay. EIA was expressed as units/mg fresh weight of biopsies from normal skin or psoriatic uninvolved and lesional skin, or units/mg normal stratum corneum or psoriatic scale. Number of samples (patients) studied in parentheses.

* Significantly different from normal epidermis and psoriatic uninvolved epidermis; $P < 0·001$ and $P < 0·05$, respectively.

† Significantly different from normal stratum corneum; $P < 0·001$.

Gel permeation chromatography

The inhibitory activity of psoriatic scale extract is shown in Figure 1. Two peaks of inhibitory activity were found with K_{av} of 0·20 and 0·41 respectively, indicating molecular weights of approximately 20 and 10 kDa. Both peaks contained inhibitory activity that was measurable in the presence of detergent. In order to investigate the possibility that the 20 kDa species is a dimer of the 10 kDa molecule, scales were processed and chromatographed in the presence of the reducing agent dithioerythritol (10 mM) to dissociate disulphide bonds. This, however, resulted

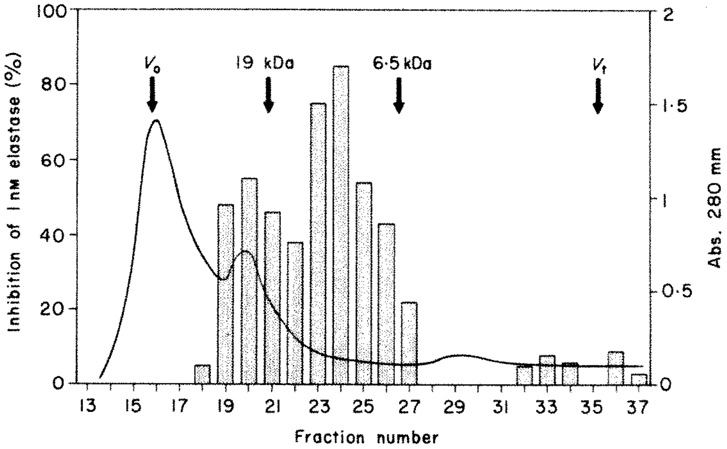

FIGURE 1. Elution profile of a Sephadex G-50 separation of psoriatic scale extract in PBS. Two major peaks of anti-elastase activity were found with approximate molecular weights of 20 and 10 kDa, respectively. Solid line: protein concentration in the eluent, bars: percentage inhibition of 1 nM elastase by 1:50 diluted fractions. V_0 = void volume, V_t = total column volume. Molecular weight markers: horse myoglobin (19 kDa) and aprotinine (6·5 kDa).

in a total loss of the inhibitory activity. When a scale extract was chromatographed under dissociating conditions using a chaotropic solvent (7 M urea in PBS) to disrupt the hydrogen bonds, no change of the elution profile (with respect to EIA) was found. To investigate the possibility that the 10 kDa species emerges from proteolytic cleavage during the extraction procedures, all processing and purification was performed in the presence of proteinase inhibitors (2 mM PMSF, 5 mM iodoacetamide, 10 mM EDTA). However, no changes in the EIA profile of the Sephadex G-50 elution were found (data not shown).

SDS-PAGE

To determine the molecular weights of the inhibitors more accurately, peak fractions of the 10 and 20 kDa species were obtained with Sephadex G-50 chromatography and pooled, concentrated, and subjected to SDS-PAGE (non-reducing conditions). The gels were stained for protein, or sliced to recover the proteins. Figures 2 and 3 show the composition of the 10 and 20 kDa fractions and the corresponding amounts of EIA. Interpolation from a curve fitted through the marker proteins confirmed that the molecular weights were approximately 10 and 20 kDa. Figure 3 shows that there is only minor contamination of the 10 kDa species with the 20 kDa fraction and vice versa. Figure 2 shows that the 10 kDa fraction is relatively pure, i.e. it only contains four major bands with molecular weights between 12,000 and 5000 kDa. Only one of the bands displayed anti-elastase activity. The 20 kDa fraction, however, shows many bands on SDS-PAGE, including keratins and other impurities. Only a weak protein band is visible at the position of anti-elastase activity as the sample had to be diluted to prevent overloading of the gel.

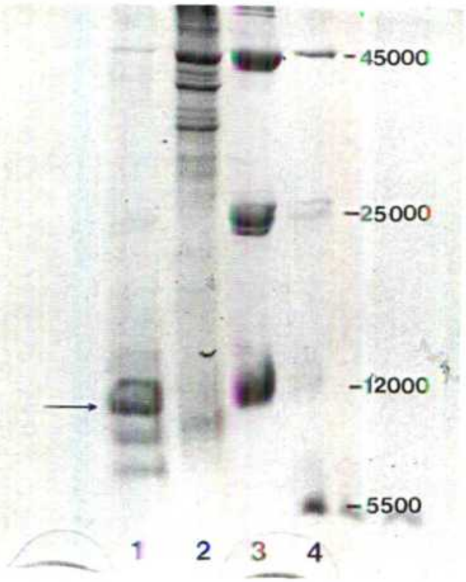

FIGURE 2. SDS-PAGE of 20 kDa and 10 kDa inhibitor obtained by Sephadex G-50 fractionation (fractions 19–21 and 23–26 from Figure 1, respectively). Lane 1, 10 kDa fraction; lane 2, 20 kDa fraction; lanes 3 and 4, marker proteins (45 kDa, ovalbumin; 25 kDa, chymotrypsinogen; 12 kDa, cytochrome c; 5·5 kDa, insulin subunit). Arrow: band coinciding with anti-elastase activity in gel slices (see Figure 4).

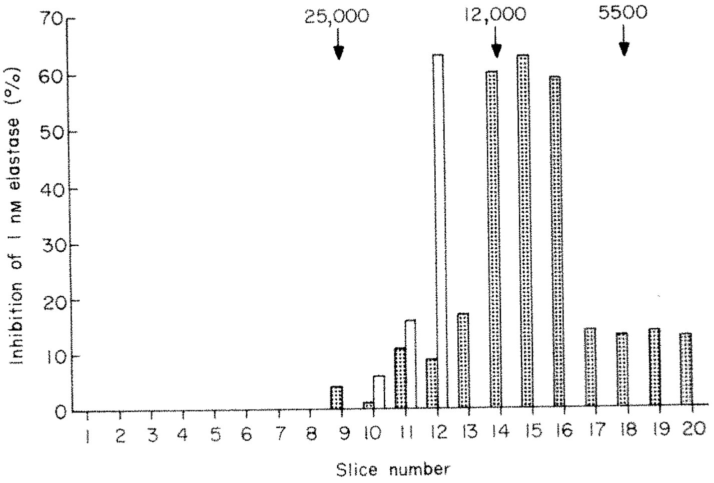

FIGURE 3. Anti-elastase activity in gel slices of SDS-PAGE separation of 10 and 20 kDa fractions from Sephadex G-50 separation (see Figure 2). Slices were extracted, concentrated and measured in the standard assay. Inhibitory activity is expressed as percentage inhibition of 1 nM elastase. Arrows with molecular weights indicate the position of marker proteins. Open bars = 20 kDa fraction; shaded bars = 10 kDa fraction.

Specificity and affinity of elastase inhibitors

Partially purified inhibitor preparations obtained by gel permeation chromatography were used to determine the inhibition constant (K_i). Figure 4 shows an inhibition curve for the 10 kDa species, using 1 nM of purified active-site titrated elastase and variable amounts of inhibitor. In view of the concave lower part of the curve, and the fact that even after prolonged incubation with an excess of the inhibitor no complete inhibition of elastase activity is found, it can be concluded that the 10 kDa species is a reversible inhibitor with a K_i far lower than the enzyme concentration used, according to the criteria posed by Bieth.[21] Assuming an equimolar stoichiometry, a K_i of approximately 5×10^{-11} M was calculated. The inhibition curve obtained for the 20 kDa species was similar to that of the 10 kDa molecule, with the same apparent K_i (not shown).

Both inhibitor preparations were tested against three other serine proteinases. Porcine pancreatic elastase was found to be inhibited in the same manner as leucocyte elastase, by both species. No activity was found against human leucocyte cathepsin G, even at high concentrations of both inhibitor and enzyme (10^{-6} M), or with a large excess of inhibitor after a prolonged incubation (60 min). The 10 kDa inhibitor was not active against bovine pancreatic trypsin. The 20 kDa preparation showed some inhibitory activity towards trypsin but confirmation awaits further purification of this species. Human α-1-PI was used as a positive control and was shown to inhibit elastase, cathepsin G and trypsin in the assay systems we used (without CTAB). Both the 10 and 20 kDa elastase inhibitor were active in the absence and in the presence of the detergent CTAB.

Source of the inhibitor

In view of the high level of EIA in psoriatic scale it seemed likely that the keratinocyte was responsible for the elastase inhibiting activity. Measurements of the EIA of cultured cells and

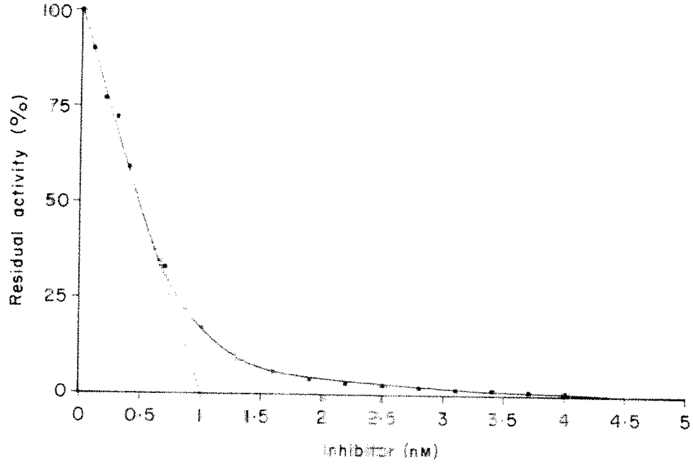

FIGURE 4. Inhibition profile of human leucocyte elastase and the 10 kDa elastase inhibitor. Purified active-site titrated human leucocyte elastase (final concentration 1 nM) was mixed with variable amounts of inhibitor preparation (obtained by gel permeation, without substantial contamination of the 20 kDa species), and the residual activity was plotted on the vertical axis. Inhibition is linear up to 30% of the original elastase activity. The amount of inhibitor yielding 50% inhibition under the assay conditions was defined as 1 unit. The intercept of the dotted line and the horizontal axis indicates the equivalence point.

inflammatory cells supported this contention and mononuclear cells from the peripheral blood did not exhibit anti-elastase activity. Similarly, in a fractionated PMN extract (in which elastase activity was eliminated by boiling for 5 min) no significant anti-elastase activity could be demonstrated (personal communication, Dr J.A.Kramps, Department of Pulmonology, University Hospital, Leiden, The Netherlands). These findings exclude the possibility that the inflammatory cells deposit the inhibitor activity in the epidermis. Large amounts of anti-elastase activity however were found in cultured human keratinocytes, as shown in Figure 5. The presence of inhibitor was specific in that cultured human skin fibroblasts, human umbilical vein

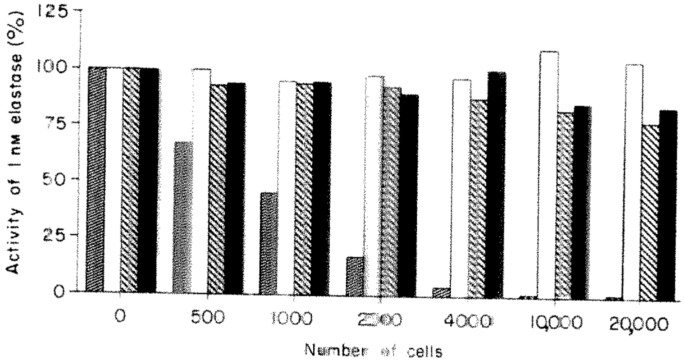

FIGURE 5. Anti-elastase activity was measured in extracts of four types of cultured cells: human foreskin keratinocytes (▩), human skin fibroblasts (□), human umbilical vein endothelial cells (▧), and human melanoma cells (■). Only keratinocytes were shown to contain significant amounts of inhibitor (approximately 1 unit per 1000 cells), whereas the other cell types were practically devoid of inhibitory activity (up to 20 000 cells tested in the assay). Vertical axis = residual activity of 1 nM elastase; horizontal axis = number of cells added in the assay.

endothelial cells and a human melanoma cell line were negative for anti-elastase activity (up to 20,000 cells tested in the assay). EIA in the cultured keratinocytes was quantitatively similar to that found in the psoriatic scale.

Induction of EIA in vivo

As already shown,[10] EIA was found in human epidermis in an experimental model which involves tape-stripping the stratum corneum. This procedure induces both hyperproliferation, as measured by recruitment of resting cells into the cycling pool,[22] and a mild transient inflammatory response as measured by the influx of PMNs 4 h after the tape-stripping.[10] EIA could be measured in the biopsies 24–168 h after tape-stripping. We characterized the EIA found in five biopsies taken at 48 h after tape-stripping and the anti-elastase activity was 43·1 ± 8·3 units/mg tissue (mean ± SD, significantly different from normal skin, $P < 0{\cdot}001$).

DISCUSSION

We have partially characterized the elastase inhibiting activity (EIA) in psoriatic epidermis and have shown that it is associated with at least two species of about 10 and 20 kDa. These inhibitors exhibit a high affinity binding of elastase and display a narrow specificity as human cathepsin G is not affected, whereas most other known elastase inhibitors are also active against this proteinase.

Proteinases derived from inflammatory cells such as PMNs or macrophages have been implicated in the pathology of various disorders such as emphysema of the lung,[5] arthritis[6] and glomerulonephritis.[7] In skin, proteases might be involved in inflammatory diseases such as psoriasis, atopic dermatitis and in various bullous dermatoses. The concentration of the main plasma proteases inhibitors with activity against PMN neutral proteases will be fairly low in the epidermis, in view of the basal lamina acting as a selective barrier for plasma proteins.[9] As extensive infiltration of the epidermis with PMNs and the formation of micropustules[23] is one of the hallmarks of the active psoriatic lesion, we investigated the protective potential of human epidermis against PMN-derived proteinases. Anti-elastase activity was found to be present in psoriatic scale. The properties of the inhibitors such as the K_i (less than 10^{-10} M), the stability and the specificity are physiologically relevant to an elastase inhibitor, although other functions, directed against hitherto unknown proteases, cannot be excluded. The fact that similar inhibitory activity could also be induced experimentally by standard injury of normal skin suggests that production of anti-elastase activity is a feedback mechanism of human epidermis to dampen the inflammatory response. This is supported by the finding of EIA in a number of inflammatory skin disorders, whereas in non-inflammatory dermatoses far less EIA is found.[9] We propose, therefore, that the elastase inhibitors act as a kind of local acute phase reactant.

As epidermis, especially when inflamed, is not a homogeneous tissue, the source of the inhibitory activity is not definitely known. However, the abundant presence of inhibitor in psoriatic scale suggests that keratinocytes are the principal source. Cultured human keratinocytes contained elastase-inhibiting activity similar, if not identical, to the activity in the psoriatic scale. The surprising observation that EIA is found in cultured keratinocytes from normal skin, whereas normal epidermis was negative, can be explained by the phenotypical similarities of cultured keratinocytes and those from lesions of psoriasis. In the culture system used the keratinocytes are hyperproliferative and express cytokeratin 16, characteristic for a hyperproliferative phenotype, as in wound healing and psoriasis.[24–26]

The inhibitor(s) reported in this paper differ from known elastase inhibitors such as the serum proteinase inhibitor α-1-PI[27] and the 10 kDa anti-leucoprotease (ALP) which also inhibits cathepsin G.[3,4] In human bronchial secretions a specific elastase inhibitor was found, which does not inhibit cathepsin G,[28] but this inhibitor has a lower molecular weight (5 kDa for the monomer), it is not inhibited by reducing agents and the dimer is dissociable in 7 M urea. It is, therefore, unlikely to be the same molecule. In view of the partial similarities to ALP we propose SKALP (skin-derived antileucoprotease) as a name for these new elastase inhibitors.

Further studies are in progress to characterize the inhibitors with respect to the molecular structure and physiological function. As both elastase inhibitors were also shown to be active against mouse, rat and rabbit elastase (data not shown), part of their function can be studied in experimental animal models of cutaneous inflammation. This will ultimately provide us with basic knowledge regarding the pathophysiological importance of proteinases and their inhibitors in human skin.

ACKNOWLEDGMENTS

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Discoid lupus erythematosus-like lesions in carriers of X-linked chronic granulomatous disease

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SUMMARY

A questionnaire was sent to 16 carriers of the X-linked cytochrome-b558 negative variant of chronic granulomatous disease (CGD). Of the 15 who answered the questionnaire and from data of one additional case, 70% reported recurrent aphthous stomatitis and 63% had recurrent skin eruptions. Five of the carriers (31%) had clinically discoid lupus erythematosus (DLE), although the histopathology was not typical and the immunofluorescence findings were negative. The infiltrate in the lesional skin of three of these patients was analysed using monoclonal antibodies against CD3, CD4, CD8, CD1a, HLA-DR, CD1c, interdigitating cells, B lymphocytes and cells of the monocyte/macrophage lineage. The immunophenotype of the infiltrating cells resembled that found in discoid lupus erythematosus.

Chronic granulomatous disease (CGD) is a syndrome in which polymorphonuclear cells (PMN) and monocytes/macrophages phagocytize micro-organisms normally, but are unable to kill these organisms intracellularly due to a deficient oxidative metabolism. This failure to produce reactive oxygen products (e.g. H_2O_2, O_2^-) leads to recurrent chronic bacterial infections of the skin, lymph nodes, liver, spleen, bone and lungs.[1]

Several subtypes of CGD have been identified and all show approximately the same clinical picture and biochemical disturbances but have distinct molecular defects. The enzyme system that produces O_2^- (NADPH oxidase) consists of several components, one of which is cytochrome-b558, and the following CGD variants are known:[2] X-linked cytochrome-b558 negative, X-linked partial cytochrome-b558 positive and autosomal recessive cytochrome-b558 positive and negative. We have studied the families of patients with the X-linked cytochrome-b558 negative variant of CGD, with particular emphasis on determining the incidence of lupus erythematosus-like skin eruptions which are often associated with the CGD carrier state.[3–12]

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METHODS

A total of 17 female carriers were studied of whom 16 completed a questionnaire.[13] The carriers were detected by subnormal hydrogen peroxide (H_2O_2) production and oxygen consumption during granulocyte stimulation, as well as by mosaicism of negative and positive cells in the nitroblue–tetrazolium slide test (NBT) after granulocyte stimulation. The questionnaire was sent to the carriers with questions as to whether there were skin and/or mucous membrane problems. Their dermatologists were asked about the type of skin eruptions, and where possible the patients were examined and skin biopsies taken. The skin lesions that were biopsied were processed for routine light microscopy, direct immunofluorescence with antibodies against IgG, IgA, IgM, C3, C1q and fibrinogen, and for the immunohistopathology of the inflammatory cell infiltrate.

A two-stage immunoperoxidase technique, with 3-amino-9-ethyl-carbazol (AEC) as staining reagent, was employed as the principal immunophenotyping procedure. The processing of tissue specimens, identification of cell types, immunoperoxidase technique and controls (neonatal thymus, lymph node, spleen) were as previously described.[14] Cryostat sections 6 μm thick were mounted on slides and, after fixation in 100% acetone, were incubated with monoclonal antibody (MAb) for 60 min in a humidified atmosphere. The sections were then incubated with a 1:20 dilution of peroxidase-conjugated rabbit anti-mouse Ig for 30 min in a humidified atmosphere. Staining was achieved by incubation of sections in a freshly prepared acetate-buffer solution (pH 4·9) that contained 50 mg AEC in 100 ml buffer, 5 ml dimethylformamide and 0·011% hydrogen peroxide. The staining reaction was visually controlled and stopped by washing in acetate buffer. The slides were fixed in 4% formaldehyde and counterstained with Mayer's haematoxylin and the sections mounted with aquamont. The MAbs used in this study are shown in Table 1. Details and references concerning the

TABLE 1. Monoclonal antibodies used in this study

MAb[15]	CD designation	Specificity
Leu 4*	CD3	Pan T lymphocytes
Leu 2a*	CD8	Suppressor/cytotoxic lymphocytes
Leu 3a + b*	CD4	Helper/inducer lymphocytes
OKT6†	CD1a	Langerhans cells
RFD-1‡	—	Interdigitating cells[16]
HLA-DR*	—	
RFD-7‡	—	Monocytes/macrophages[16]
RFD-9‡	—	Epitheloid cells[16]
OKM 5†	—	Monocytes/macrophages
M241§	CD1c	Indeterminate cells[17]
Leu 14*	—	B lymphocytes
Leu 7* (HNK-1)	—	Natural killer cells
B1¶	—	Peripheral B lymphocytes

* Becton Dickinson, Sunnyvale, CA, U.S.A.
† Ortho-Pharmaceuticals, Rariton, New Jersey, U.S.A.
‡ Gift from L.W.Poulter, London.
§ Gift from R.W.Knowles, New York.
¶ Coulter Laboratories, Hialeah, FL, U.S.A.

specificities of the MAbs can be found in earlier studies.[15-17] Epidermal cells were quantified as the number of positive cells present per epidermal length. The percentages of positive cells in the dermal infiltrates were estimated as the number of cells surrounded by plasma membrane stained with the MAbs per 100 counterstained nuclei.

Neutrophils were isolated and treated with the nitroblue-tetrazolium slide test (NBT),[18] while oxygen consumption and hydrogen peroxide formation were measured as previously described.[19]

RESULTS

Fifteen of the 16 questionnaires sent were returned and the data were analysed together with that from one additional carrier. The carriers varied in age from 15 to 60 years (mean 34 years). The results of the NBT slide test after granulocyte stimulation (PMA 1 ng/ml) (normal value 98–100% positive cells), and O_2 consumption (normal value: $8{\cdot}9 \pm 1{\cdot}4$ nmol $O_2/10^6$ neutrophils/min) and H_2O_2 production (normal value $4{\cdot}3 \pm 1{\cdot}1$ nmol $H_2O_2/10^6$ neutrophils/min) after granulocyte stimulation (by PMA) are shown in Table 2.[20] The NBT slide test varied from 3 to 94% positive cells (mean 47%), the O_2 consumption from 9 to 85% (mean 47%) of the mean of the normal values (in nmol/10^6 granulocytes/min) and the H_2O_2 production from 1 to 60% (mean 33%) of the mean of the normal values (in nmol/10^6 granulocytes/min).

Eleven carriers (70%) reported that they had a recurrent aphthous stomatitis and 10 (63%) suffered from relapsing skin eruptions that were provoked by sunlight in seven. Three carriers complained of Raynaud's phenomenon and three had recurrent skin infections. These three

TABLE 2. Clinical details concerning the carriers of X-linked CGD

Patient no.	Age	NBT-test*	O_2 consumption†	H_2O_2 production†	Aphthous stomatitis	Skin problems	Special features
1	49	40	38	26	+	+	
2	23	15	9	2	+	+	Recurrent infections
3	35	57	ND	39	+	+	
4	20	44	ND	ND	+	–	
5	15	64	ND	ND	–	–	
6	45	62	ND	ND	–	+	
7	37	53	37	58	+	+	Raynaud's phenomenon
8	37	ND	67	50	–	–	
9	34	94	85	39	–	–	
10	34	37	ND	29	+	+	Raynaud's phenomenon
11	36	68	56	55	+	–	
12‡	27	12	12	13	–	–	Recurrent infections + immune comp. nephritis
13	60	ND	ND	18	+	+	
14	49	67	65	60	+	+	
15	16	47	51	35	+	+	Raynaud's phenomenon
16	24	3	ND	1	+	+	Recurrent infections

* Percentage formazan positive cells.

† Percentage of the mean normal value (nmol/10^6 granulocytes/min).

‡ Published elsewhere ref 20.

ND, not done.

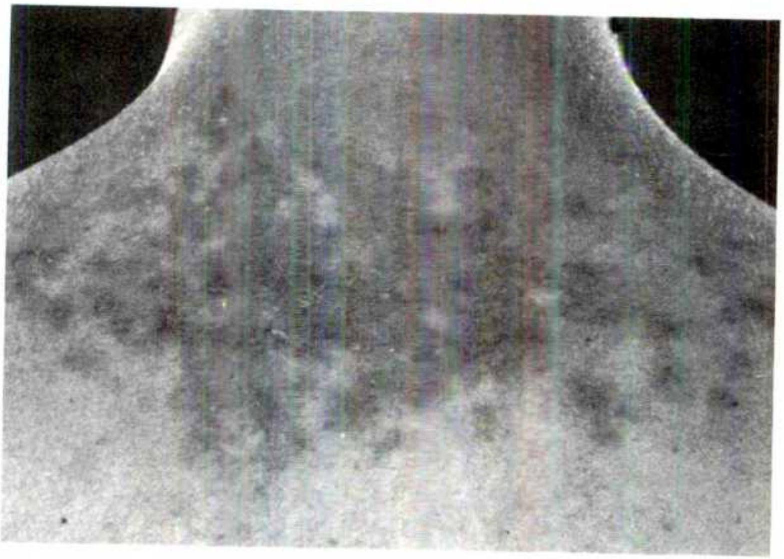

FIGURE 1. Sun-exposed area of skin on the back showing erythematous and scaly lesions with focal hypopigmentation and atrophy (patient No. 10).

patients showed the lowest values for the NBT slide test, O_2 consumption and H_2O_2 production in the whole group, being 15, 9 and 2% respectively in patient No. 2; 12, 12 and 13% respectively in patient No. 12; and a NBT of 3% and a H_2O_2 of 1% in patient No. 16.

In five of the 10 carriers with relapsing skin eruptions a clinical diagnosis of discoid lupus erythematosus (DLE) was made. This was based on the presence of the skin lesions (Fig. 1). The skin biopsies of these five CGD carriers all showed some of the histological features of DLE[21] (Table 3), but not sufficient to allow a definite diagnosis (Fig. 2). Direct immunofluorescence in three of the biopsies showed some granular deposits of C3, and in one a linear deposition of fibrinogen at the basement membrane zone.

TABLE 3. Histopathological findings consistent with DLE found in the skin biopsies of patients showing DLE-like skin lesions

	Patient no.				
	1	2	7	10	16
Follicular plugging	−	+	+	+	+
Hyperkeratosis	−	+	−	+	−
Epidermal atrophy	−	+	−	+	+
Hydropic degeneration of basal layer	−	+	−	+	+
Lymphocytic infiltrates in the dermis	−	+	+	+	+
Dilated blood vessels	+	−	−	−	+
Dermal infiltrate around follicles	+	+	+	+	±

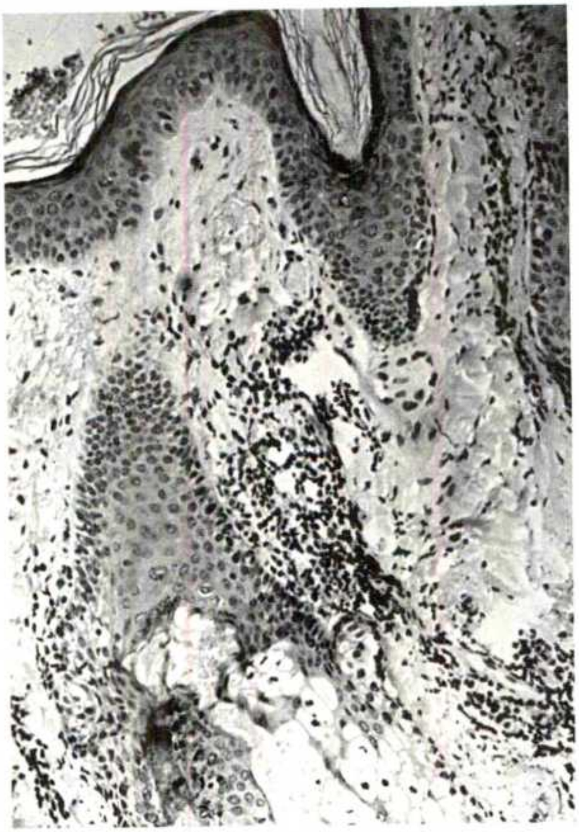

FIGURE 2. Biopsy of DLE-like lesion showing slightly atrophic epidermis with follicular plugging and focal areas of hydropic degeneration. A lymphocytic infiltrate is present around the superficial blood vessels and hair follicles. (Haematoxylin and eosin × 28.)

The immunophenotyping of the cellular infiltrates in the skin lesions of three patients using MAbs are shown in Table 4. In the epidermis, 1–5 $CD3^+$ cells were found in 0·3 mm length of the epidermis. Most of the cells of the dermal perivascular infiltrate were T lymphocytes ($CD3^+$). A majority of these cells (1–3, 3–10 and 0–4 cells per 0·3 mm epidermis in the epidermal compartment and 44, 63 and 57% positive cells in the dermal compartment, respectively) were found to be $CD4^+$. Only sporadic $CD8^+$ cells were found in the epidermis (0–1 per 0·3 mm) and these cells formed 25, 33 and 37% of the dermal infiltrate. In these three patients, the CD4/CD8 ratio was always more than 1 (1·2, 2·5 and 1·7). Few dendritic cells were found using anti-CD1a, RFD-1 and CD1c antibodies, the number in the epidermis in normal skin in our laboratory being 4·2 ± 0·5 $CD1a^+$ cells per 0·3 mm epidermal length, while in the CGD carriers with DLE-like skin lesions the mean was 2·6 positive cells per 0·3 mm in lesional skin. Strong staining for HLA-DR of the keratinocytes was observed in the lesional skin. B lymphocytes ($B1^+$) were not seen either in the epidermis or in the dermis. Natural killer cells (Leu 7^+) were found to be present in small numbers (less than 1%) in the dermal infiltrate. Cells staining with MAbs of the monocyte/macrophage lineage were rare in the dermis and mainly absent in the epidermis. OKM 5^+ cells formed 10–14% of the dermal infiltrate and in one patient 0–5 OKM 5^+ cells per 0·3 mm were seen in the epidermis.

TABLE 4. Distribution of immunocompetent cells in lesional skin

	Patient no.					
	7		10		16	
	Epidermis*	Dermis†	Epidermis*	Dermis†	Epidermis*	Dermis†
Leu 4 (CD3)	2–5	73	1–3	58	1–6	68
Leu 2a (CD8)	0–1	25	0–1	37	0–1	33
Leu 3a + b(CD4)	3–10	63	1–3	44	0–4	57
OKT6(CD1a)	0–3	0	0–3	0	0–2	0
RFD-1 (HLA-DQ?)	1–4	20	0–3	Diffuse	0	Aspecific
HLA-DR	IC-substance	Diffuse	IC-substance	Diffuse	IC-substance	Diffuse
RFD-7	0	Sporadic	0	1	0	1
RFD-9	0	0	0	0	0	Sporadic
OKM-5	0–5	14	0	?	0	10
M241	0	0	0	0	0	0
Leu 7	0	1	0	1	0	0
B1	0	0	0	0	0	0

* Values in number of cells per 0·3 mm.
† Values in percentage of total infiltrated cells.
IC, intercellular.

DISCUSSION

In our study 70% of the X-linked CGD carriers investigated reported that they suffered from aphthous stomatitis and 31% had discoid lupus erythematosus-like skin lesions. These findings agree with a previous report on skin and mucous membrane lesions in carriers of CGD[8] but in contrast with another report,[9] we could not find a relationship between the results of the NBT slide test and skin condition in our population. In three carriers, only the rate of skin infections was related to very low values for the NBT, H_2O_2 production and O_2 consumption. The mean of the NBT slide test results in carriers with skin problems is 38% in our population, whereas in a previous report of 15 carriers[9] it was only 19%. Carriers without skin problems had a mean of 68% positive cells in the NBT slide test,[9] whereas in our population the mean was 56%.

The results of immunophenotyping in our three patients who had DLE-like skin lesions were compared with those of patients with DLE.[22-26] The majority of cells in the dermal infiltrates were $CD3^+$. The CD4/CD8 ratios varied from 1 to 3. $CD4^+$ cells in the epidermis were reported only once,[23] but $CD8^+$ cells were observed in the studies that specified the occurrence of these cells in the epidermis.[23,24] $CD1a^+$ cells were described as being less frequent than normal in the epidermis,[23,25] and one study noted that the dendrites of these cells appeared smaller than usual.[23] Only a few $CD1a^+$ cells were observed in the dermal infiltrate.[23,24] The presence of B lymphocytes in the dermal infiltrate was investigated in three studies[22,24,25] and B cells were found only rarely. The similarity of the findings in the DLE-like skin lesions in CGD carriers with those in patients with DLE suggests a common pathogenesis. In CGD carriers, biologically active remnants may act as neoantigens and induce an autoimmune process resulting in DLE-like skin lesions.

Two hypotheses have been formulated concerning the origin of lupus erythematosus. The membrane attack hypothesis[27] suggests that undefined factors lead to activation of the complement-system, with subsequent deposition of the membrane attack complex and generation of complement-mediated damage. The antibody-dependent cellular cytotoxicity hypothesis[28] suggests that specific antibodies bind to an antigen on the target cell surface, and non-specific effectors attach to the target-bound antibody by Fc receptors. This Fc-receptor activation leads to target-cell lysis by release of toxic oxygen products, proteolytic enzymes or lymphotoxin by cytotoxic cells. The fact that direct immunofluorescence showed neither linear depositions of immunoglobulins nor complement in the basal membrane region in our CGD carriers does not seem to support the first hypothesis, in which the local deposition of immunoglobulins, antigens and complement components is considered necessary to generate a membrane attack complex. The results of this study are more in accordance with the second hypothesis. Functional studies of lymphocytes derived from lesional tissue should reveal whether or not these cells are indeed cytotoxic. If so, it is possible that in CGD carriers, the failure of the abnormal phagocytes to degrade cells damaged for example by UV-light leads to T-cell-mediated cytotoxicity. Patients with DLE should be asked about the occurrence of severe chronic skin infections in their families.

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Multinucleate cell angiohistiocytoma: an acquired vascular anomaly to be distinguished from Kaposi's sarcoma

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SUMMARY

Multinucleate cell angiohistiocytoma is a newly described benign vascular condition that usually arises on the extremities of women over the age of 40 as discrete grouped violaceous erythematous papules, often mimicking Kaposi's sarcoma. Of 10 patients, nine were women aged between 37–66 (average 51·5) years at the onset of their condition. The legs, and in particular the calves and thighs, were the commonest sites to be involved. However, in three patients the papules were confined to the back of the hands, and the male patient had lesions across the front of the chest. Bilateral lesions occurred in four patients. Histologically, the salient features were proliferation of capillaries and small venules at the level of the subpapillary plexus and the mid dermis, in association with prominent connective tissue cells and larger angulated multinucleate cells. Detailed histochemical and immunocytochemical studies have not elucidated the histogenesis of the multinucleate cells that seem to be a characteristic feature of this condition.

We describe the clinicopathological spectrum of multinucleate cell angiohistiocytoma (MCAH) in 10 patients with particular emphasis on the differential diagnosis from that of Kaposi's sarcoma (KS). The histogenesis of the disorder was also investigated by cell marker studies. Some details of five of these cases have been previously reported.[1,2] The clinical picture in these patients was that of slowly progressive, grouped, vascular lesions arising on the extremities and, in one case, on the chest. Two further cases of MCAH have been studied using a variety of cell markers and with electronmicroscopy.[3]

This is, to our knowledge, the first detailed report of a substantial number of cases of this rare disorder.

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CASE REPORTS

Details of the ten patients are summarized in Table 1. All patients, except case 9, had been recognized as MCAH as a result of biopsies referred by outside dermatologists and pathologists. The diagnosis of case 9 (a patient of Dr Geoffrey Hunter at Adelaide, Australia) had been suspected as a result of a biopsy which was referred to us for confirmation. Clinical photographs were available of all the patients except cases 9 and 10. They were all Caucasian adults, eight living in the U.K., one in Australia (case 9) and one in Canada (case 10).

Clinical features

All the patients had six or more vascular papules, except patient No. 8, who only had two papules. Most of the papules varied in size from 2 to 10 mm and were discrete, well circumscribed lesions with an ovoid shape. They were generally a dull-red colour with variable violaceous or brownish tints. They were soft to firm in consistency and were not tender. The lesions were irregularly scattered over the area of the affected skin (Figs 1 and 2) but occasionally some of the papules were closely grouped or appeared to have coalesced (Figs 1 and 2). Some of the lesions in patient No. 7 were annular in shape. The history of those patients for whom full

TABLE 1. Clinical details of 10 patients with MCAH

Patient no.	Sex and age at onset	Sites affected	Duration (years)	No. of biopsies	Suspected clinical diagnosis prior to biopsy
1.	F 54*	Back of left hand	3·5	4 × FF	KS, granuloma annulare, multiple histiocytoma
2.	F 50*	Right calf and inner thigh, left calf	6–7	2 × FF 1 × PLP + CR	Lichen planus, pseudo-KS
3.	M 37*	Front of upper chest	16	5 × FF 1 × PLP 1 × PLP + CR	Sarcoidosis, lymphocytoma, KS, Spitz naevus
4.	F 47*	Back of both hands	7	5 × FF 1 × PLP + CR	?Lichen planus ?Insect bites
5.	F ?65	Right calf	2·5	1 × FF	KS
6.	F 62	Both calves	4	1 × FF	—
7.	F 52*	Below left knee	7	2 × FF 1 × PLP + CR	Sarcoid, granuloma annulare
8.	F 66	Left inner thigh	6 months	1 × FF	Insect bite reaction, vascular tumour
9.	F ?	Both knees	1	1 × FF	Granuloma annulare, necrobiosis lipoidica
10.	F 38	Fingers	1	1 × FF	Lupus erythematosus, sarcoid, erythema elevatum diutinum

* Patients seen personally by the authors and additional biopsies obtained.
FF, routine formalin-fixed tissue.
PLP, periodate–lysine–paraformaldehyde fixation.
CR, cryostat section prepared from unfixed tissue.

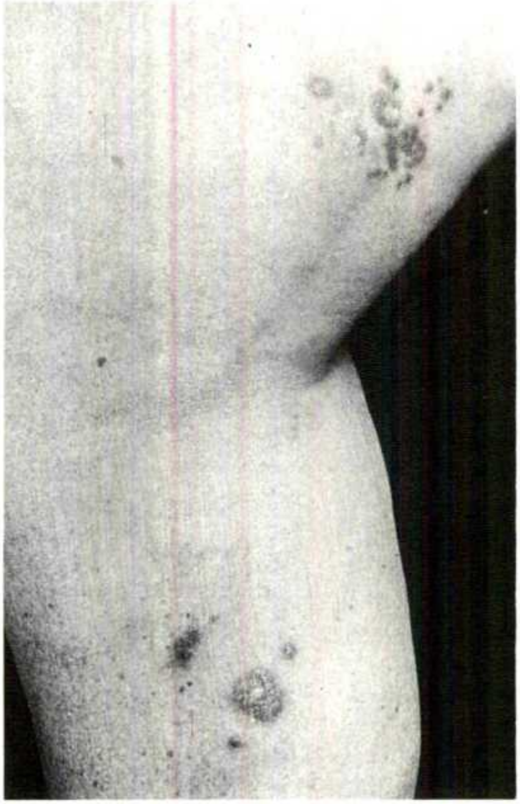

FIGURE 1. Case No. 2. Grouped lesions affecting two separate areas.

details were available, indicated that the lesions had developed insidiously and were symptomless. Spontaneous resolution of lesions was rare but was recorded in patient No. 4.

Patient No. 2 was unusual in that three separate sites were involved. Initially, five to six lesions developed on the right calf which persisted, and later new grouped lesions developed on the middle of the right inner thigh, widely separated from the original lesions (Fig. 1). Three to four years later several papules developed on the opposite calf. She had always been in good health and had five children; she gave no history of venous thromboses and there were no obvious signs of venous stasis.

Patient No. 3 presented in 1974 with a 2-year history of developing seven to eight brownish, violaceous papules arranged in an approximately linear distribution across the front of the upper chest (Fig. 2). There was no history of preceding trauma. Several new papules developed over the course of the next 4 years, during which time several of the larger lesions were biopsied. In 1979 the patient was admitted to hospital and the remaining papules, with the surrounding skin, were totally excised. In 1984, when he was recalled for follow-up, there was no evidence of recurrence. However, two new papules later developed which, at the patient's request, were re-excised early in 1988. The histological findings were similar in all the specimens.

The histories of the patients have provided no clue as to any provoking factor or of any preceding underlying vascular abnormality. Patient No. 7 had had long-standing ulcerative

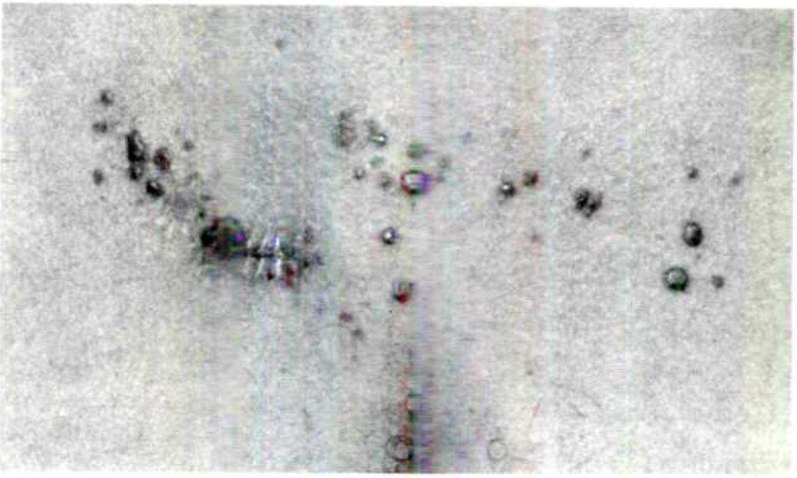

FIGURE 2. Case No. 3. Multiple lesions in horizontal distribution across the front of the chest.

colitis and 15 years previously she had a saggital sinus thrombosis. No history of serious medical disorders were recorded in the other cases.

HISTOPATHOLOGY

Twenty-eight biopsies or surgical specimens were available for study. The majority of the tissues had been routinely fixed in formaldehyde saline and embedded in paraffin wax prior to the preparation of sections. Four specimens were fixed in freshly prepared chilled periodate–lysine–paraformaldehyde (PLP) for 2 h prior to a modified rapid paraffin-embedding procedure, designed to allow optimal antigen preservation for immunocytochemical procedures.[4] Cryostat sections of unfixed tissue were also available from three specimens for enzyme histochemistry. Techniques, sources of reagents, specificity and results of immunocytochemical and enzyme histochemistry are summarized in Table 2. Besides routine haematoxylin and eosin-stained serial sections, the following standard stains were also studied: periodic acid Schiff (PAS), alcian blue, Perl's reaction for blood pigment, orcein for elastica, and the chloracetate esterase reaction to demonstrate mast cells.

Routine histology

Apart from some variability in the number of multinucleate cells and the extent of the vascular changes, the histology was remarkably consistent in all of the specimens. In the affected segments of the skin, increased numbers of small blood vessels were present in the upper and mid-dermis in small groups with individual vessels set a little apart from each other (Fig. 3). Most of the vessels showed narrow lumina, but a few were ectactic (Fig. 4). There was little or no discernible increase in the number of larger arteries or veins in the lower dermis deep to the lesion. Tortuosity or dilation of capillaries in the dermal papillae above the area of vascular proliferation was also slight or absent. The predominant vessels identified in the lesions were either capillaries or small venules occurring in approximately equal numbers. The venules were surrounded by a variable layer of smooth muscle cells or pericytes. The endothelial cells in the venules were generally prominent with ovoid nuclei indenting the lumina of the vessels. One in

10 or less of the vessels appeared to be arterioles. (Arterioles can usually be distinguished from venules by the fact that their surrounding perithelial cells are much more prominent, possessing elongated curved banana-shaped nuclei, while in contrast their endothelial cells are flatter with smaller condensed nuclei than in venules. In vessels of this small size, the internal elastic lamina is too poorly developed in arterioles to be of diagnostic value). A sparse to moderately dense lymphocytic infiltrate surrounded vessels in some of the specimens. Occasionally there was an admixture of plasma cells and neutrophils. Lymphatic vessels were not prominent.

Besides the variable presence of perivascular inflammatory cells, there was a general increase in the number of interstitial connective tissue cells and larger 'histiocytic' cells scattered randomly between and at a distance from the vessels. There appeared to be no direct relationship between the vascular and connective components of MCAH. In approximately two-thirds of the specimens multinucleate cells were also prominent and these had between three and eight nuclei. Their nuclei were closely aggregated or overlapping (Fig. 5). They possessed less cytoplasm than ordinary foreign body type giant cells, and many were bizarre in shape with irregular scalloped outlines (Fig. 6). In a minority of the lesions the multinucleate cells were sparse but could usually be demonstrated in serial sections. Haemosiderin-containing macrophages were sparse or absent, except in two specimens obtained from lesions on the lower leg; foamy macrophages were not seen. The multinucleate cells were not significantly PAS positive. The alcian blue stains indicated a minor degree of accumulation of acid mucopolysaccharide in some of the lesions. Normal elastic tissue was well preserved within lesions and the epidermis and the skin appendages showed no obvious abnormalities.

Immunocytochemistry and histochemistry

The lesional vessels were labelled in a similar manner to capillaries and venules in normal skin. In particular, nearly all vessels were positive with the monoclonal antibody PAL-E[5] (Fig. 7) and with factor VIII-related antigen (FVIII-RA), a finding consistent with the capillary and venular nature of the great majority of the vessels. Lymphatic-like, PAL-E and FVIII-RA-negative vessels, such as commonly occur in early (macular) KS,[6] were not seen. With the universal endothelial cell markers EN4,[4] BMA 120[7] and *Ulex europaeus* agglutinin-1[4] the lesional vessels were labelled as intensely as normal vessels of a similar size. BMA 120 labelling was generally more intense in the small blood vessels than in larger vessels with a multi-layered muscle coat. Most of the smaller MCAH vessels were also alkaline phosphatase positive. Most of the small lymphocytes sleeving vessels were labelled with UCHL1, suggesting they were T cells, but occasional small foci of L26 positive cells were detected in some specimens, indicating a B-cell component.

Most of the mononuclear interstitial cell population of MCAH was labelled for Factor XIIIa (FXIIIa)[8] (Fig. 8), lysozyme and $\alpha 1$ antitrypsin. However, generally only a few cells (less than 5%) were positive with MAC 387[9] and for S100 protein. In the specimens showing blood pigment accumulation, increased numbers of MAC 387 positive cells were found in the same areas. There was a slight increase in numbers of mast cells, as detected by the chloracetate esterase reaction. In contrast, the multinucleate cell population could not be labelled with any of the preceding techniques, but were strongly vimentin positive. The multinucleate cells showed some reactivity with adenosine triphosphatase, non-specific esterase and acid phosphatase of a similar intensity to that found in the other interstitial cells. Enhanced reactions with the latter two hydrolases were not apparent, as might be expected if the multinucleate cells in MCAH were similar to foreign body type multinucleate giant cells.

TABLE 2. Immunocytochemical and enzyme histochemistry of biopsies from MCAH patients

	Main specificity	Dilution and type of antibody		Source	Results in lesions
Immunocytochemical histochemistry					
FVIII RA*	Vascular endothelium: capillaries and veins arteries > lymphatics, megakaryocytes	MAb PAb	1/75 1/100	Dako¶	Positivity confined to vessels
BMA 120^{7}	Vascular endothelium, sweat glands canaliculi	MAb	1/10	Behringer**	Positivity confined to vessels and sweat glands
EN4^{3}	All vascular and lymphatic endothelium	MAb	1/1‡	(a) gift of Dr. CJF Spry (b) Sanbio	Positivity confined to vessels
PAL-E*	Vascular endothelium: capillaries and venules > > arterioles. Lymphatics negative	MAb	1/1‡	Sanbio††	90% of vessels positive
*Ulex europaeus** Agg-1 (UEA-1)	All vascular and lymphatic endothelium. Cell membranes of many epithelial structures	UEA-1 Anti-UEA-1 PAb	1/100000 1/100	Sigma‡‡ Dako	Positivity confined to vessels, epidermis and appendages
FXIIIa^{8}†	Dermal and subcutaneous 'fixed' connective tissue cells	PAb	1/400	Behringer	Most lesional interstitial cells positive. Multinucleate cells negative
S100 Protein*	Nerves, Langerhans' cells, melanocytes: sweat glands ±	PAb	1/500	Dako	As per normal structures. Occasional positive cells in infiltrate. Multinucleate cells negative
MAC 387†	Monocytes and monocyte-derived macrophages, skin appendages and 'stimulated' epidermis	MAb	1/2000	Dako	An occasional positive cell in infiltrate. Multinucleate cells negative. Epidermis positive above lesion
Vimentin	Connective tissue cells, nerves, blood vessels and most inflammatory cells	MAb	1/100	Dako	All cells in infiltrate and vessels positive, including the multinucleate cells

Lysozyme†	Macrophages and neutrophils	PAb	1/2000	Dako	Interstitial cells positive, but multinucleate cells negative
α-1-antitrypsin†	Macrophages and neutrophils	PAb	1/1000	Dako	Interstitial cells positive, but multinucleate cells negative
UCHL-1	70% of T lymphocytes	MAb	1/10	Dako	Majority of lesional lymphocytes
L26	Majority of B lymphocytes	MAb	1/200	Dako	Occasional focus of positive lymphocytes
PKK1	Pan keratin marker	MAb	1/100	Lab Systems	No positive dermal cells
Enzyme histochemistry		Histochemical techniques			
Alkaline phosphatase	Capillaries, myoepithelial cells. Fibroblasts in scars. Hair papillae	Gomori's calcium phosphate method. Burstone azo-dye simultaneous coupling method§		Sigma	Positivity confined to small, not dilated vessels. Connective tissue cells negative
Adenosine triphosphatase	Most cells. Strongest reactions in blood vessels, smooth muscle cells and Langerhans cells	Wachstein and Meisel lead method magnesium activated§		Sigma	Positive in vessels, interstitial cells and multinucleate cells
Non-specific esterase	Stongly positive in histiocytes and macrophages and granular cell layer of the epidermis	Holt and Withers indoxyl acetate method§		Sigma	Positive in interstitial cells and multinucleate cells
Acid phosphatase	Strongly positive in histiocytes and macrophages and granular cell layers of the epidermis	Burstone azo-dye coupling method§		Sigma	Positive in interstitial cells and multinucleate cells

* Pre-digestion with pronase (Type XXIV Sigma 0·01% in for 15 min).

† Pre-digestion with trypsin (Difco 0·1% in 0·1% $CaCl_2$ for 10 min).

MAb, monoclonal antibody.

PAb, Polyclonal antibody.

‡ PLP fixed sections.

§ Cryostat sections.

¶ Dako, High Wycombe, Bucks, U.K.

** Behringer, Middlesex, U.K.

†† Sanbio, Bradsure biological, Leics, U.K.

‡‡ Sigma Chemical Co., Poole, Dorset, U.K.

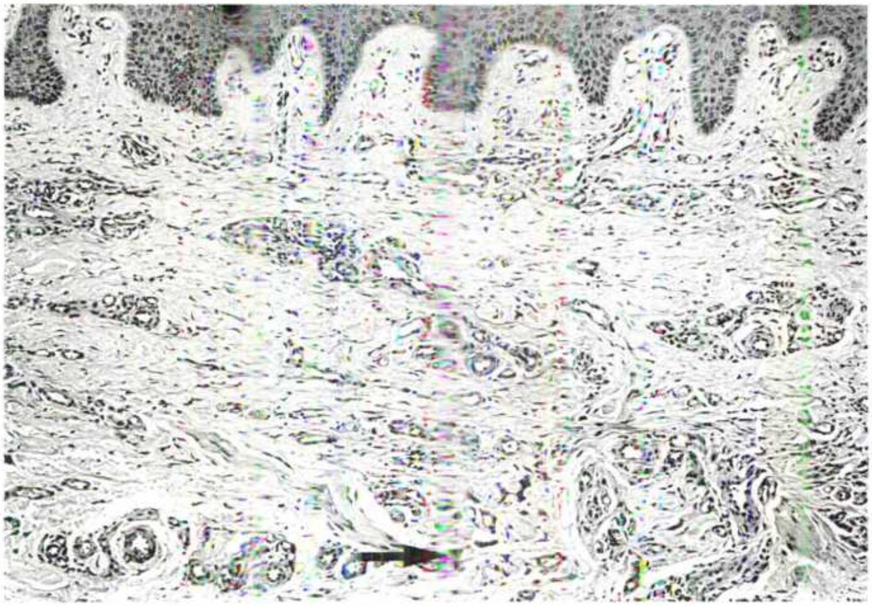

FIGURE 3. Case No. 2. Increased numbers of capillaries and venules in the upper and mid dermis. Note angulated multinucleate cells below centre of picture (arrow). H&E × 60.

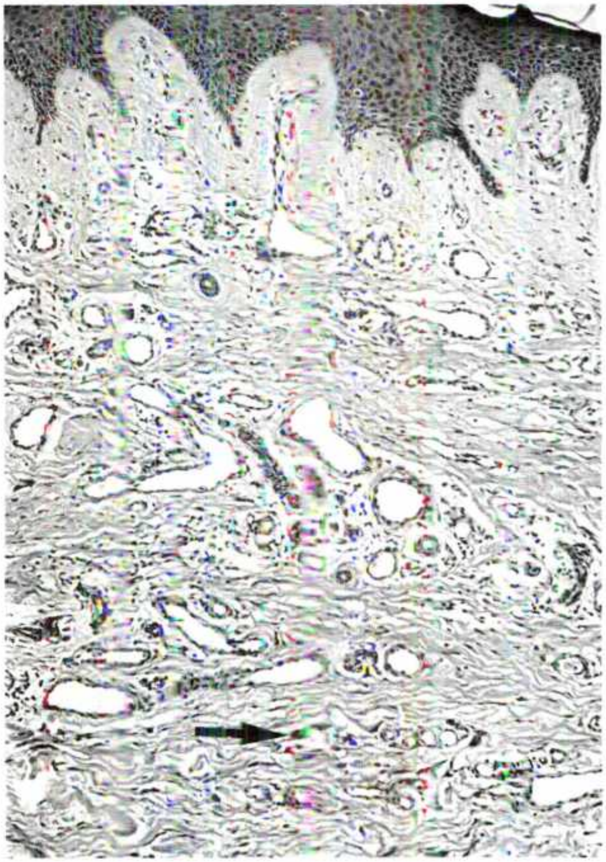

FIGURE 4. Biopsy case No. 5. Dilated capillaries and venules with mild inflammatory cells. Multinucleate cells (arrow). H&E × 60.

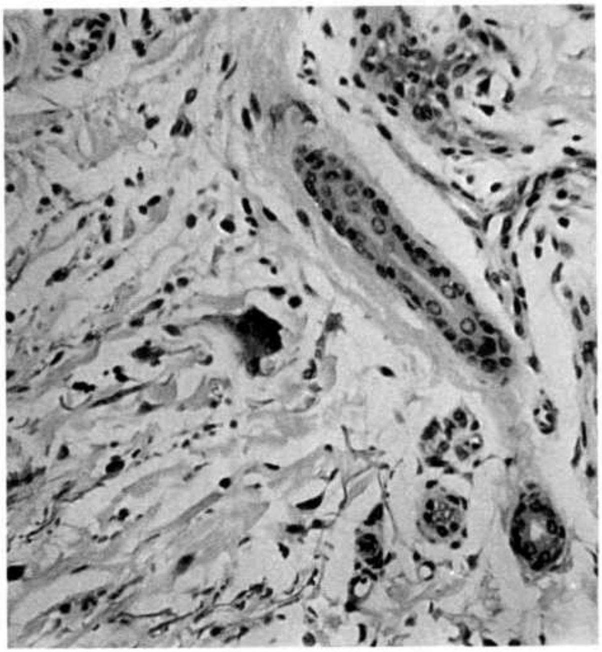

FIGURE 5. Case No. 2. Variable sized multinucleate cells in the vicinity of a sweat duct and small blood vessels. H&E × 400.

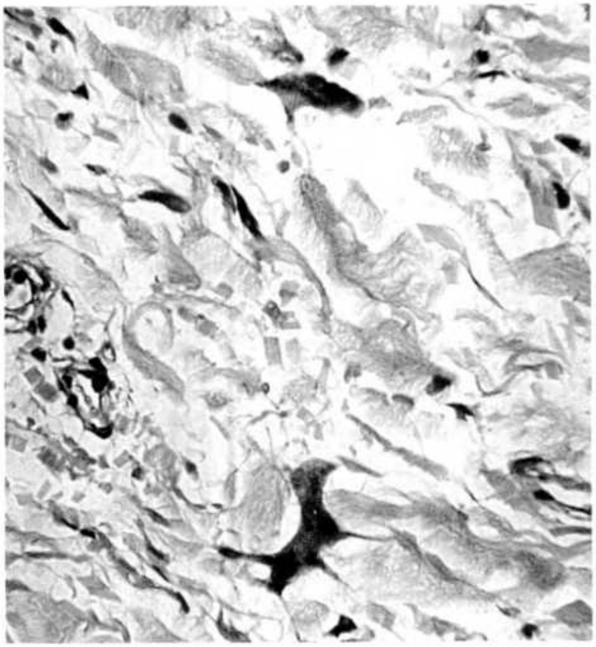

FIGURE 6. Case No. 2. Multinucleate cells showing clumped nuclei and scalloped outlines. H&E × 400.

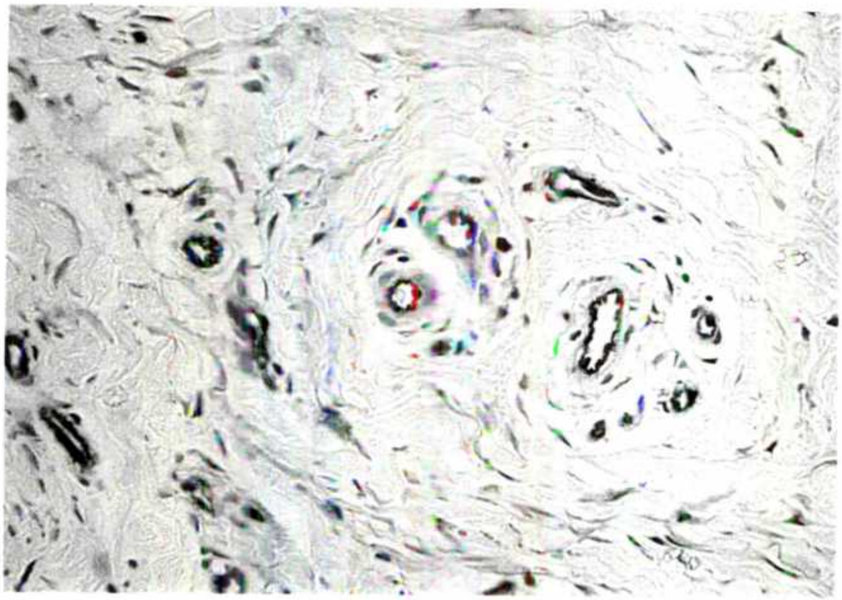

FIGURE 7. Case No. 5. PLP fixed tissue. Labelling of vascular endothelium with monoclonal antibody E6, lightly counterstained with haemalum. (×250).

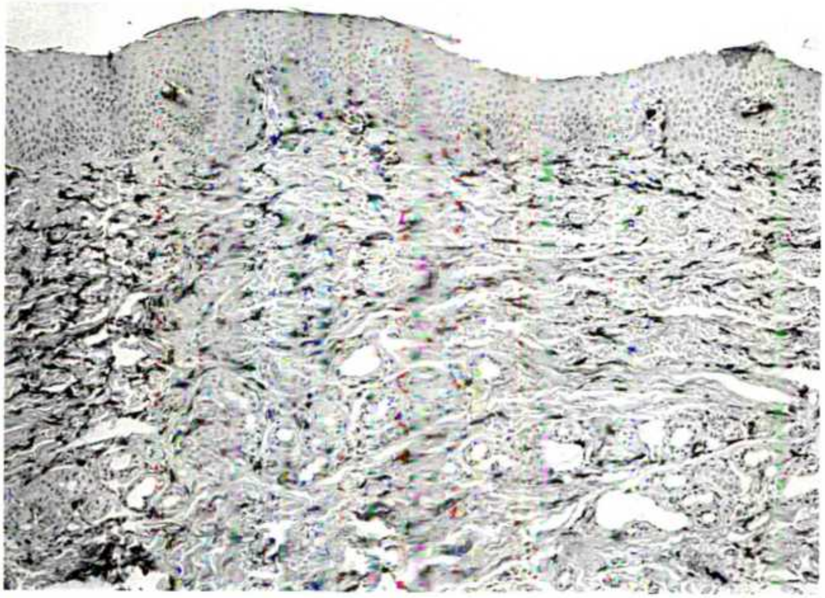

FIGURE 8. Case No. 5. Labelling for FXIII-a showing a diffuse increase of positive cells in the lesional skin. (H&E ×40).

DISCUSSION

Our studies indicate that MCAH is a distinct clinicopathological entity. In our series, the commonest presentation with grouped vascular lesions on the lower legs of older patients gives a clinical picture that could easily be mistaken for KS. However, the predominance of females with MCAH and the differences in the pathology exclude the possibility that MCAH is an unusual variety of KS. The back of the hands appears to be the next most common site, and it is of interest that one of the patients described by Smolle *et al.*[3] had lesions on the back of one hand and on both knees. However, other sites can be affected; patient No. 3 in our series had lesions on the chest and the first case of Smolle *et al.*[3] had bilateral papules affecting the face and eyelids. The vascular nature of MCAH was not always clinically apparent at presentation prior to biopsy (Table 1), because the lesions were usually only dull-red or brownish in colour. Generally the lesions of MCAH are smaller than those of classical KS in the elderly and large bruise-like macules, plaques or nodules do not develop. The feet and ankles are very frequently affected in KS but not to date in MCAH. Lesions on the calves of older patients also suggest the diagnosis of pseudo KS—lesions that arise due to high local venous pressure. However, the absence of purpura at the ankles in MCAH or of evidence of varicosities elsewhere in the affected limb is against this possibility. Also the histopathology of pseudo KS differs greatly from that of MCAH. In pseudo KS, tortuosity and 'cork screwing' of papillary vessels, pronounced deposition of blood pigment in macrophages and the presence of thick-walled blood vessels in the mid and lower dermis are essential features, but these do not occur in MCAH.

We are not aware of any other specific vascular anomaly that reproduces the histological findings that have been outlined in MCAH. The nearest histological equivalent is that of an unusual atrophic type of dermal histiocytoma in which vessels are abnormally prominent but the histocytes are sparse. In such lesions multinucleate cells are often present. Three solitary histiocytomas of this type have been studied for comparison with MCAH. Features distinguishing these from MCAH are evidence of a reduction in thickness of the dermis at the site of the histiocytoma and a denser population of histiocytes (dermal dendrocytes). Usually in this type of histiocytoma, histiocytes demonstrate a 'curlicue' pattern between collagen bundles, a feature not encountered in MCAH.

With regard to the histogenesis of MCAH, we are uncertain as to whether connective tissue changes or the vascular proliferation is the primary abnormality. Smolle *et al.*[3], in their discussion concerning the histogenesis, have mainly concentrated on the connective tissue changes and in particular they discuss the similarities with and differences from fibrous papules of the nose. The multinucleate cells in both conditions closely resemble each other. Most authors take the view that fibrous papules of the nose represent a connective tissue disorder,[9,10] but others[11-13] still believe that most cases represent a special form of involuting cellular naevus. A recent study[14] has shown that the predominant cells in fibrous papule of the nose are FXIIIa positive and vimentin positive, but their larger multinucleate cells were only occasionally positive for FXIIIa. A small percentage of the papules showed groups of S100 protein positive cells that could be taken as evidence of residual naevus cells, suggesting a naevoid origin. However, S100 positive grouped cells suggestive of an intradermal or Spitz naevus have not been encountered in MCAH.

FXIIIa appears to be an excellent marker of normal fixed dermal connective tissue cells and which has emphasized the dendritic character of the cells.[8,15] Approximately 50% or more of the cells comprising common histiocytomas are FXIIIa positive. As the main connective tissue cells in MCAH are also FXIIIa positive this helps to justify the use of the descriptive term 'angiohistiocytoma' in the title of MCAH positive.

Increased numbers of FXIIIa positive cells occur in many inflammatory disorders.[15] The increased numbers of FXIIIa cells in early lesions of AIDS-associated KS have been cited as an argument that initially KS is non-vascular in origin.[16] It has been shown, however, that in the classical lesions of KS the characteristic spindle cells are FXIIIa$^-$. The probability is that the resident FXIIIa positive dendritic cells in the dermis proliferate in response to a variety of inflammatory and possibly immunological stimuli and therefore care must be taken in interpreting their presence in various lesions.

It remains puzzling as to why bizarre multinucleate cells occur so frequently in MCAH and what their relevance may be. Similar multinucleate cells can be found in atrophic vascular histiocytomas that differ from Touton-like or other giant cells that occur in ordinary xanthomatous or haemosiderotic histiocytomas. Another observation of possible relevance is that multinucleate cells similar to those of MCAH can often be encountered in lesions of lichen simplex chronicus in the papillary dermis in association with other chronic inflammatory cells.[17] Such multinucleate cells have, we feel, been inappropriately referred to as 'endothelial' giant cells.[18] Bizarre multinucleate cells of a similar type can also be found in chronic radiodermatitis and in profuse numbers in necrobiotic xanthogranuloma.[19] Our immunocytochemical results give no clear indication as to the derivation of these cells, in particular they do not label with MAC 387 (a marker of monocyte derived macrophages),[9,15] as do many foreign-body-type giant cells in granulomas. Also the multinucleate cells of MCAH do not label with FXIIIa—but nor do most other multinucleate cells.[15] It is proposed that the multinucleate cells of MCAH and similar cells in lichen simplex and radiodermatitis represent degenerate or 'effete' connective tissue cells or macrophages that, under conditions of prolonged chronic stimulation, have failed to divide after mitotic division and have finished up as 'sterile' and functionless cells.

We conclude that MCAH is a specific chronic inflammatory disorder of unknown cause whose main histological features are vascular hyperplasia and increased numbers of unusual connective tissue cells. Multinucleate cell angiohistiocytoma must now be added to the growing list of conditions—pseudo KS, progressive lymphangioma,[20] spindle cell haemangioma,[21] tufted angioma,[22] angiosarcoma[23] and epithelioid angiomatosis[24] that enter into the differential diagnosis of Kaposi's sarcoma.

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ADDENDUM IN PROOF

Since the submission of this paper one of us (R.C.) has characterized FXIIIa dermal dendritic cells in normal adult and neonatal skin using double immunofluorescence labelling techniques.[25] The immunophenotype of this connective tissue population suggests that they are resident bone-marrow derived non-Langerhans cells that share epitopes with mononuclear phagocytes (monocyte/macrophages).

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Renal function after long-term low-dose cyclosporin for psoriasis

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SUMMARY

Two groups of patients receiving cyclosporin A (CyA) for psoriasis had their renal function assessed by measurement of glomerular filtration rate (GFR) and effective renal plasma flow (ERPF). The first group comprised 13 patients who had taken low dose (average 3 mg/kg per day) CyA for an average period of 2·5 years. Seven of the 13 had a normal GFR of 108 (77–121) ml/min (median; range). In the other six patients the GFR was low at 63 (50–77) ml/min and CyA was discontinued for periods ranging from 3 to 17 weeks. The GFR rose in all six patients, to 79 (60–91) ml/min; this change was significant ($P < 0·05$). The six patients restarted CyA because their psoriasis recurred and after a mean interval of 15 weeks the GFR had fallen in all six to 63 (46–80) ml/min ($P < 0·05$) and the ERPF decreased from 339 (231–414) ml/min to 244 (177–321) ml/min ($P < 0·05$). In the second group of 11 patients measurements were made prior to starting CyA and after taking CyA for a mean of 9 weeks. The GFR fell in eight out of 11 subjects, the GFR for the 11 patients being 117 (72–128) ml/min before taking CyA and 97 (51–122) ml/min after CyA ($P < 0·02$). The ERPF was measured in nine of the 11 patients and fell in seven of the nine. The ERPF for the nine patients before CyA was 490 (296–642) ml/min and for the 11 patients after CyA was 410 (195–543) ml/min ($P < 0·01$). This study shows that impairment of renal function is reversible in patients with psoriasis after long-term dosage with CyA. However, this impairment may occur after short- as well as long-term treatment.

Nephrotoxicity has been recognized as a side-effect of cyclosporin A (CyA) treatment since the discovery that it was an immunosuppressive agent and its subsequent use in organ transplantation. The exact nature of the nephrotoxicity is uncertain. In renal transplants it may be difficult to distinguish between nephrotoxicity due to CyA, ischaemia, or repeated episodes of graft rejection. In non-kidney transplantation, pre-operative renal impairment is not unusual and there may be concomitant therapy with other nephrotoxic drugs. Thus, there is controversy

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as to whether the renal changes are due primarily to CyA. However, nephrotoxicity due to CyA has been reported in non-transplant patients, with uveitis and other autoimmune diseases.[1,2]

It has been claimed that nephrotoxicity, whether in the transplant or non-transplant patients, is likely to be dose related.[3] However, no studies to date have been reported on low dosages of CyA. CyA given in comparatively low dosage has now been shown to be an effective treatment in clearing psoriasis,[4,5] and in maintaining this clearance over a long term.[6] As the main side-effect of CyA is nephrotoxicity it is important to determine if CyA taken at low dosage over a long term is safe for patients with psoriasis. We have measured glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) radioisotopically to assess changes in renal function.

METHODS

Patients

Two groups of patients with severe psoriasis were studied.

Group 1. This comprised 13 patients (eight female and five male; age range 27–78 years, mean age 50 years) who took CyA for a mean of 2·5 years (range 20–37·3 months). The mean dose of CyA was 3 mg/kg per day throughout the study (range 1–5 mg/kg per day). The GFR was determined in all 13 patients and the ERPF in seven. In six of the 13 patients the GFR was low and in these CyA was stopped for periods ranging from 3 to 17 weeks and the GFR and ERPF repeated. CyA was restarted and both tests carried out again after an average period of 15 weeks (range 7–26 weeks).

Group 2. This comprised 11 patients (two female and nine male; age range 31–61 years, mean age 45 years). The GFR was measured in 11 and the ERPF in nine patients prior to starting CyA. Both tests were repeated in all 11 patients after they had taken CyA for an average period of 9 weeks (range 4–18). The average dose of CyA during this period was 3 mg/kg per day (range 2–4).

Measurement of GFR and ERPF

GFR (ml/min) was measured by single injection of Cr-51 EDTA with blood samples taken every 30 min for 2–4 h;[7] the zero time blood activity and $T\ 1/2$ (min) was calculated graphically, giving an error of ± 6 ml/min. The normal value GFR was defined as 105 ± 26 ml/min at 30 years, 98 ± 23 ml/min at 50 years and 78 ± 24 ml/min at 70 years.[8] The ERPF (ml/min) was measured by a simultaneous injection of I-125-*o*-iodohippurate and from blood samples taken at 30, 45 and 60 min. The value at 44 min was calculated graphically,[9] giving an error of ± 17 ml/min. The filtration fraction (FF) was calculated by dividing GFR by ERPF, the normal value being between 0·20 and 0·30. Comparisons of GFR and ERPF, on and off CyA, were made using Wilcoxon match-pairs signed-rank test.

RESULTS

Group 1

The GFR for the group of 13 patients taking long-term CyA was 77 (50–121) ml/min (median; range). Seven of the 13 patients had a normal GFR for their age of 108 (77–121) ml/min. The other six patients had a low GFR for their age of 63 (50–77) ml/min. In these six patients the drug was discontinued and renal function tests repeated after periods of 3–17 weeks, depending

TABLE 1. Changes in glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and filtration fraction (FF) in patients with low GFR (corrected for age) on CyA, off CyA and after restarting CyA. Comparisons made on median values using (Wilcoxon match pairs signed rank test)

			Taking CyA					Discontinuation of CyA				Recommencement of CyA			
Patient no.	Age	Sex	Duration (weeks)	Dose mg/kg	GFR	ERPF	FF	Discontinuation (weeks)	GFR	ERPF	FF	Duration (weeks)	GFR	ERPF	FF
1	47	F	87	3·2	72	322	0·22	4	91	414	0·20	13	76	321	0·24
2	36	F	132	2·9	77	ND	—	3	86	348	0·24	16	80	291	0·28
3	58	F	135	2·9	71	278	0·26	4	89	349	0·26	26	65	250	0·26
4	67	F	129	3·6	50	ND	—	4	60	249	0·24	20	46	185	0·25
5	78	M	136	3·0	56	ND	—	3·5	72	329	0·22	7	57	239	0·24
6	72	F	83	2·2	50	ND	—	17	64	231	0·28	7	61	177	0·34
Mean	59·7		117	3·0	63*	300	0·24	4	79*	339†	0·24	14·8	63*	244†	0·25

* GFR on CyA and off CyA $P < 0·05$.

* GFR off CyA and restarting CyA $P < 0·05$.

† ERPF off CyA and restarting CyA $P < 0·05$.

ND, not done.

TABLE 2. Changes in glomerular filtration rate (GFR), effective renal plasma flow (ERPF) and filtration fraction (FF) in patients measured before and soon after starting CyA for the first time

Patient no.	Age	Sex	Before CyA GFR	Before CyA ERPF	Before CyA FF	Taking CyA Duration (weeks)	Taking CyA mg/kg	Taking CyA GFR	Taking CyA ERPF	Taking CyA FF
1	61	M	72	296	0·24	18	2·0	51	195	0·26
2	38	M	127	642	0·20	4	3·5	122	540	0·23
3	37	F	90	378	0·24	4	3·5	92	324	0·28
4	51	F	117	ND	—	9	3·5	94	410	0·23
5	41	M	121	437	0·28	8	3·0	91	282	0·32
6	31	M	125	616	0·20	12	3·5	112	543	0·21
7	57	M	105	ND	—	16	2·5	97	478	0·20
8	34	M	111	490	0·23	4	4·0	118	51	0·23
9	52	M	95	358	0·27	8	3·0	95	390	0·24
10	34	M	117	563	0·21	6	3·0	104	417	0·25
11	61	M	128	605	0·21	10	2·0	99	402	0·25
Mean	45		117*	490†	0·23	9	3·0	97*	410†	0·24

Comparisons made on median values using Wilcoxon match pairs signed rank test.

* GFR before and after CyA $P < 0{\cdot}02$.

† ERPF before and after CyA $P < 0{\cdot}01$.

on the recurrence of the psoriasis. CyA was then reintroduced because of the relapse in the psoriasis. The results of changes in GFR and ERPF are shown in Table 1. The GFR rose significantly on stopping the CyA but returned to approximately the same value as after long-term CyA, on reintroduction of the drug. The FF remained within normal limits, except in patient No. 6 when she recommenced CyA.

Group 2

The results of GFR and ERPF before and after 4–18 weeks treatment with CyA are shown in Table 2. There was a significant fall in both GFR and ERPF after taking CyA but the FF remained constant.

DISCUSSION

This study shows that impairment of renal function is reversible in patients with psoriasis after long-term treatment with CyA taken at a relatively low dose. In those patients with a low GFR, there was a rise in the GFR when CyA was discontinued. As the values for the GFR and ERPF were not determined prior to starting CyA, it is not known whether the GFR and ERPF had returned to their pretreatment levels when the CyA was stopped. The GFR was at the lower range of normal values for the patients' ages even after stopping CyA, and there may have been an even greater rise in the GFR if it had been possible to discontinue CyA for longer periods, but this was not practical because of the relapse of the psoriasis in all six patients. In addition, three of the six patients (Nos 4, 5, 6, group 1) with a low GFR, were the oldest of both groups 1 and 2, and it is possible that older patients are more susceptible to the nephrotoxic action of CyA. It has been reported that reversibility of impaired renal function due to CyA is less likely in patients over 60 compared to those under this age.[1] Also one of the six patients (No. 4) was taking a NSAID, which is known to have a synergistic nephrotoxic effect with CyA.

It has been known since the initial use of CyA in organ transplantation that nephrotoxicity is dose related.[3] Permanent renal damage has been found in non-transplant individuals who were receiving relatively high doses (initially 10 mg/kg per day) for uveitis,[2] but there may have been disease-associated renal dysfunction. The advantage of studying patients with psoriasis is that it is not associated with any known renal dysfunction and responds to a low dose of cyclosporin. The results of the present study in psoriasis also supports the hypothesis that the primary site of nephrotoxicity is one of vasoconstriction of the afferent glomerular arterioles, as both the GFR and ERPF tend to fall proportionally.[10,11] Until the long-term nephrotoxic effects of CyA in the treatment of psoriasis are known it is advisable to carry out radioisotope studies of renal function prior to commencement of CyA. Serum creatinine and clearance estimations are not sufficiently accurate to detect mild to moderate cases of renal dysfunction which subsequently may be incorrectly attributed to CyA.

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Cyclosporin A levels in suction-blister fluid of patients with psoriasis treated systemically

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SUMMARY

Oral cyclosporin A (CyA) is highly effective in the treatment of psoriasis. The long-term use is limited by dose-dependent side-effects, and the local concentration of CyA is a determining factor in treatment. The concentration of CyA in suction-blister fluid (SBF) and in whole blood was assessed using a polyclonal radioimmunoassay (RIA). This was carried out in patients with psoriasis following a single dose of CyA and while on adequate oral treatment with the drug. Peak blister fluid levels ranged from 32 to 170 ng/ml, and whole blood levels from 1250 to 2540 ng/ml. CyA trough levels (12 h following taking the drug) in the blister fluid ranged from 22 to 113 ng/ml, and in whole blood from 148 to 935 ng/ml. The trough concentrations in SBF were approximately 10% of whole blood trough levels.

Systemic cyclosporin A (CyA) has been found to be highly effective in the treatment of psoriasis.[1,2] It is suggested that the drug acts locally in the skin, since a significant improvement has been obtained with intradermal injection of CyA into plaques of psoriasis.[3] However, topical application of CyA was insufficient to control psoriasis.[4] As yet the pharmacokinetics and target specificity of CyA in the skin are not fully understood, though it is generally accepted that the main target in psoriasis is activated lymphokine-secreting T helper–inducer cells.[5] As suction-blister fluid (SBF) is considered to represent skin interstitial fluid, in this study we determined the concentration of CyA in SBF in patients who had psoriasis and who were in remission while under oral treatment with this drug.

METHODS

Patients

Ten male patients (aged 27–77 years) with stable, severe chronic plaque psoriasis and with a PASI score of 26–42 were selected for oral treatment with CyA (Sandimmun®) following failure

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to improve with other treatments for their psoriasis. These patients had not previously received CyA, and had not been treated with topical agents in the 2 weeks before or with systemic drugs in the 4 weeks prior to the study. Informed consent was obtained from all the patients.

Experiment 1

In four patients the CyA concentration–time curves were determined in whole blood and in SBF obtained from the non-lesional skin following the administration of a single dose of CyA. Three of these patients (Nos 1, 2 and 3) had an oral dose of 5 mg/kg body weight and patient No. 4 received a single dose of 10 mg/kg. The SBF and whole blood were collected at timed intervals following administration of the drug.

Treatment with oral CyA was continued until remission was induced, and then the dose was reduced to the lowest level that would maintain a remission of 85–95%, as previously described.[6] Individual oral doses ranged from 1·8 to 4·3 mg/kg per day, and under this maintenance treatment the CyA concentration–time curves were again recorded in whole blood and in SBF obtained from non-lesional skin.

Experiment 2

In the other six patients a remission was induced, and the dose of CyA adjusted, and this ranged from 2·5 to 5·6 mg/kg per day. The trough levels of CyA were determined under maintenance treatment in whole blood and in SBF obtained from previously involved skin. To determine whether the skin lesions would recur at these sites, the dose of CyA was reduced slightly in several of these patients.

Collection of SBF and whole blood samples

SBF (0·1 ml) and 3 ml heparinized venous blood were collected at the time of the oral administration of CyA and approximately every 15 min during the first 2 h, and every half-hour over the next 8 h, and at 12, 24 and 72 h. Samples were stored at room temperature.

Suction blisters were produced using a Dermovac® device[7] on the volar surface of the forearm. Blisters were raised on non-lesional skin (experiment 1) or on previously involved skin (experiment 2). Blisters were not raised on lesional skin, due to fragility of the blister roofs. A negative pressure of 300 mmHg at room temperature resulted in blister formation within 60–90 min without any apparent haemorrhage. The blisters to be harvested in the first 2 h of the experiment were raised before the actual administration of CyA, and due to an inter-individual variation in time of blister formation, the sampling schedule could only be approximated. Whole blood CyA levels rather than plasma levels were measured.[8]

Drug assay

Whole blood and SBF CyA levels were determined by a polyclonal Sandoz radioimmunoassay kit, in which the ^{3}H-tracer was replaced by a ^{125}I-tracer. CyA concentrations determined in a pilot study in SBF appeared to be much lower than in whole blood. The usual sample dilution of 1:51 as indicated in the Sandoz manual was therefore reduced to 1:10. To maintain standard conditions, SBF dilutions were prepared with 1:51 diluted (0 time) blank whole blood.

RESULTS

Experiment 1

The CyA concentration–time curves for SBF and whole blood following a single oral dose

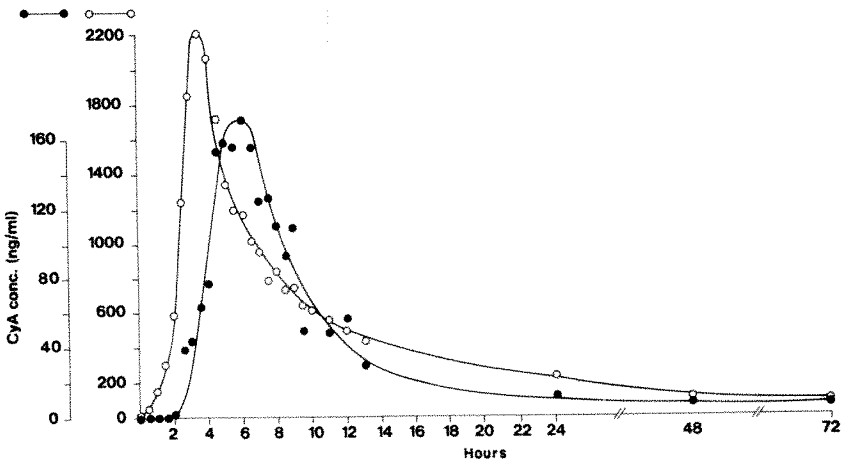

FIGURE 1. Concentration–time curve in suction blister fluid (●) and whole blood (○) after a single oral dose of 5 mg/kg cyclosporin A in a representative patient (No. 1).

TABLE 1. Single dose and steady state CyA concentrations in SBF (obtained from non-lesional skin) and whole blood

		Peak levels		Trough levels		
					SBF	
Patient no.	Dose (mg/kg per day)	Blood (ng/ml)	SBF (ng/ml)	Blood (ng/ml)	(ng/ml)	% of blood
	Single dose					
1	5	2200	170	504	56	11·1
2	5	1360	54	148	12	8·1
3	5	742	32	274	22	8·0
4	10	2540	133	542	78	14·4
	Steady state					
1	2·9	1320	103	427	64	13·6
2	4·3	1370	170	236	23	9·7
3	1·8	1640	153	790	71	9·0
4	4·0	1250	102	371	44	11·6

showed a large inter- and intra-individual variation which was not dose-dependent. A representative curve is shown in Figure 1. The peak and trough concentrations of CyA are shown in Table 1. Although falling CyA blood concentrations could be fitted to a triexponential equation, our study was too small to allow more conclusions to be made on the pharmacokinetics. The single dose and steady-state data are therefore grouped together. Peak whole blood levels (Table 1) ranged between 742 and 2540 ng/ml, and SBF peak levels ranged between 32 and 170 ng/ml. Peak SBF levels were reached 2–4 h after peak levels in whole blood. From about 7 h following oral dose a dynamic equilibrium was reached between SBF and whole blood levels (Fig. 1). SBF levels ranged from 5 to 14·4% of the whole blood levels. At 12 h after

TABLE 2. Trough CyA concentrations in SBF (obtained from healed skin) and whole blood from patients under maintenance treatment

Patient no.	Dose (mg/kg per day)	Blood (ng/ml)	SBF (ng/ml)	SBF % of blood
5	5·2	558	48	8·6
6	5·0	935	102	10·9
7	3·0	887	113	12·7
8	4·7	660	58	8·8
9	5·6	605	60	9·9
10	2·5	240	20	8·3

administration of CyA, trough levels in whole blood ranged from 148 to 790 ng/ml, and in SBF from 12 to 78 ng/ml.

Experiment 2

Trough blood levels in six additional patients on maintenance treatment with CyA ranged from 240 to 935 ng/ml, and SBF levels from 20 to 113 ng/ml (Table 2). Inter-individual variations again appeared unrelated to the administered dose. The Wilcoxon test did not show any statistical difference between trough levels in experiments 1 and 2.

DISCUSSION

The efficacy of cyclosporin A in the treatment of psoriasis is established, but the mode of action of this drug is still a matter of debate.[5] The localized effect of CyA when injected intradermally in psoriasis indicates that it acts in a concentration-dependent manner in the skin. Patients with psoriasis treated with CyA at a dosage of 14 mg/kg per day, showed in keratotome biopsies taken from lesional skin, that the concentration of CyA in the epidermis was 1–3 μg/ml.[9] These levels approximated to the whole blood levels, and were high enough to suppress proliferation of T cells, and have also been shown to suppress the proliferation of keratinocytes and fibroblasts cultured in serum-free medium.[9,10] However, there was no significant antiproliferative effect with these levels of CyA when fibroblasts and keratinocytes were cultured in a serum-containing medium.

While concentrations in the keratotome biopsies represent the actual CyA concentration in the epidermis, it is possible that some part of the CyA is located where the drug is not pharmacologically active. Using radiolabelled CyA-loaded human Burkitt lymphoma cells, only a slow loss of the intracellular radiolabel was observed when these cells were resuspended in a medium free of CyA.[11] Removal of the extracellular CyA however rapidly restored normal lymphocyte function.[12–14]

The CyA is believed to reach target cells in the skin through the interstitial fluid and direct cell-to-cell transfer of CyA was not found (M.B.M. Teunissen, personal communication). The CyA concentrations in interstitial fluid can be assessed using skin-blister techniques.[15–17] In experiment 1, we found that from 7 to 8 h after the administration of CyA there was a dynamic equilibrium in the levels of CyA between SBF and whole blood. The SBF levels were approximately 10% of the whole blood levels (range 5–14·4%). The SBF peak levels in patients with psoriasis in remission on treatment with CyA ranged from 102 to 170 ng/ml, and the SBF trough levels from 20 to 113 ng/ml. These concentrations appeared to be sufficient to maintain control of the psoriasis. These levels are also compatible with inhibition of T cells, but insufficient to affect proliferation of keratinocytes and fibroblasts *in vitro*.

Our finding that SBF levels of CyA are substantially lower than whole blood levels was expected, and reflects the distribution of drug between blood cells and plasma, and the ratio of lipoproteins in interstitial fluid and plasma. About 50% of the whole blood CyA is located in the plasma compartment, and is mainly associated with lipoproteins.[18-20] The concentrations of high and low density lipoproteins in SBF are 25 and 16% respectively of those in plasma.[21] Lower serum concentrations of lipoproteins enhance the inhibition of the *in vitro* growth of human T cells, fibroblasts and keratinocytes by CyA.[9,10,22] The intracellular uptake of CyA was enhanced two- to threefold when keratinocytes were cultured in a serum-free medium.[9] The uptake of CyA by lymphocytes was enhanced when the drug was complexed with low-density lipoproteins.[23] Several recent studies have shown the protective effect of an elevated concentration of plasma lipid *in vivo*, and elevated triglyceride levels were associated with a reduced efficacy and distribution of the drug despite high serum levels.[24] Also, high cholesterol levels gave protection against hypertension and nephrotoxicity.[25] These findings are related to the drug being very lipophilic.

It is not yet known whether the extracellular SBF levels or the intracellular accumulated levels of CyA are more relevant to its *in vivo* effects. Further investigations are indicated to establish the location of the drug in the skin following oral administration, and also to determine the penetration of new and more effective topical formulations of CyA for the treatment of psoriasis.

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Cyclosporin A treatment of systemic sclerosis

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SUMMARY

Ten patients with severe progressive systemic sclerosis were treated with cyclosporin A at dosages of 1·5–7·5 mg/kg per day: two patients showed a marked improvement with softening of the involved skin; in one patient there was healing of persistent digital ulcers; one patient showed initial improvement followed by a marked exacerbation; one patient was slightly improved; two patients were unchanged. The clinical results correlated well with levels of serum aminoterminal propeptide of type III procollagen. In the eight patients completing treatment, side-effects such as slight tremor and a transient increase in the serum creatinine and blood pressure were either absent or reverted to normal when the dosage was decreased, except one case who had to be treated with anti-hypertensive drugs.

Systemic sclerosis (SS) or systemic scleroderma is a disease involving the skin and the connective tissue of other organs. There is progressive fibrosis in this disorder for which there is, as yet, no adequate therapy although penicillamine, which affects cross-linking of collagen, may be of some benefit.[1,2]

Different immunological mechanisms have been implicated in the pathogenesis of the disease.[3,4] Although autoantibodies against nuclear and cellular antigens are highly specific for SS, there is still no evidence that they are directly involved in its pathogenesis. Attention has been directed to cell-mediated immunity, and an elevated ratio of T4/T8 lymphocytes has been detected in SS[5] together with augmented T-helper cell function.[6] Since interleukin 1 modulates fibroblast metabolism[7] and antigen-stimulated T cells can increase collagen synthesis,[8] we considered cyclosporin A (CyA) as a treatment for SS.[9] We present our studies on the treatment of SS with CyA including investigations on serum aminoterminal propeptide of type III procollagen (PIIINP), that we previously found to be a marker of fibrogenesis in this disorder.[10]

METHODS

Three males and seven females with severe progressive SS were treated with CyA at doses of 1·5–7·5 mg/kg per day. All patients fulfilled the criteria for scleroderma of the American

Rheumatism Association.[11] Three had diffuse cutaneous SS, while the others had progressive localized cutaneous SS. The duration of disease varied from 1 to 30 years. Four patients were treated within the first 2 years of their disease. All but two had an abnormal pulmonary function test and oesophageal involvement was demonstrated in all the cases apart from one, by radiomanometry. Five of the patients had kidney biopsies performed prior to treatment, and three of these had a second biopsy after 1 year of treatment. None of the patients had hypertension, abnormal creatinine clearance, or proteinuria at the start of treatment. CyA blood levels were monitored initially at weekly and later at up to 6-weekly intervals. Levels of PIIINP were measured by radioimmunoassay based on the human propeptide;[12] this was done before and during treatment in six patients, and only once in the other four.

RESULTS

The clinical results are summarized in Table 1. In two patients (Nos 1 and 9) there was a marked regression of the skin fibrosis with softening and no further progression of the disease was noted. One patient (No. 10) had complete healing of her persistent, painful digital ulcers. In two other patients (Nos 2 and 4) an initial clinical improvement was observed, but this was followed in one by a pronounced exacerbation after approximately 6 months of treatment. Three patients were unchanged, and two patients discontinued treatment after 4 and 6 weeks due to neurological side-effects. Both were started on a relatively high dose (7 and 7·5 mg/kg). Side-effects in other patients, such as slight tremor and an increase in the serum creatinine and blood pressure, were either absent or disappeared when the dose of CyA was decreased, except in one patient who had to be treated with antihypertensive drugs. None of the patients went into renal failure.

All five pretreatment kidney biopsies were normal, but in one patient after a year of treatment the biopsy showed mild CyA-associated arteriolopathy,[13] and this resulted in the patient stopping therapy with CyA. Another post-treatment biopsy showed similar changes and the treatment of this patient was changed to prednisone and azathioprine. No significant visceral improvement occurred with CyA treatment in these cases.

The levels of PIIINP are shown in Table 1 and Figure 1. Four of seven patients had pretreatment values above the reference range (1·7–4·2 μg/l)[7] and one had a borderline value (4·2 μg/l). All the patients monitored before and during treatment had a significant initial decrease in levels of PIIINP (paired *t*-test, $P = 0{\cdot}002$). However, in one patient this was followed by a significant increase on developing myositis. In the patient with the highest pretreatment values in which a significant decrease in PIIINP was accompanied by good clinical improvement, PIIINP had gradually increased from 3·7 μg/l to 8·1 μg/l during 6 months prior to treatment with CyA, while the patient was on prednisone.

DISCUSSION

The results of this study agree well with those of Frances *et al.*[14] who studied skin collagen in patients with scleroderma before and after CyA therapy. CyA treatment was associated with a clinical improvement in two of their four patients and with a clinical and biochemical improvement in the other two. Their results were also consistent with the data of Fleischmajer *et al.*[15] who suggested that an increase in collagen III occurs during the initial inflammatory stage of SS. France *et al.*[14] also found an increased ratio of radiolabelled CNBr peptides of type III to type I collagen in SS skin with a non-significant decrease after treatment. One of their patients

TABLE 1. Clinical response and initial PIIINP values in patients with systemic sclerosis treated with cyclosporin A

Patient no.	Sex	Age (years)	Type*	Duration of disease	Progression of exacerbation	Clinical response	Significant side-effects	Dosage (mg/kg)		Duration of treatment (months)	Results† of kidney biopsy		PIIINP (µg/ml)	
								Highest	Maintenance		Before	After	Before treatment	First during treatment
1	F	78	DS	2	++	Good	None	7	2	16	—	—	6·2	5·8
2	M	33	LS	12	+	Slightly improved	None	7·5	3	12	—	—	—	4·0
3	F	28	LS	21	+	Unchanged	Renal	4	2·5–3	12	N	P	4·2	3·2
4	F	44	LS	1	+++	Worsened	None‡	4	3	12	N	(P)‡	6·0	4·9
5	M	49	LS	30	+	Unchanged	None	3·5	1·5	12	N	N	3·8	2·8
6	F	41	LS	17	++	No evaluation	Neurological	7	—	1½	—	—	—	7·4
7	F	66	LS	16	+	No evaluation	Neurological	7·5	—	1	—	—	3·7	—
8	F	16	DS	11	+	Unchanged	None	5·5	5	5	—	—	—	5·8
9	M	64	DS	2	+++	Good	20% increase in serum creatinine	4·5	2·5	4	N		8·1	6·4
10	F	25	LS	2	+++	Good	None	4·5	4·5	3	N		5·5	4·4

* DS = diffuse cutaneous systemic sclerosis; LS = localized cutaneous systemic sclerosis.

† N = normal biopsy; P = pathological biopsy.

‡ This patient had a doubtful post-operative pathological kidney biopsy.

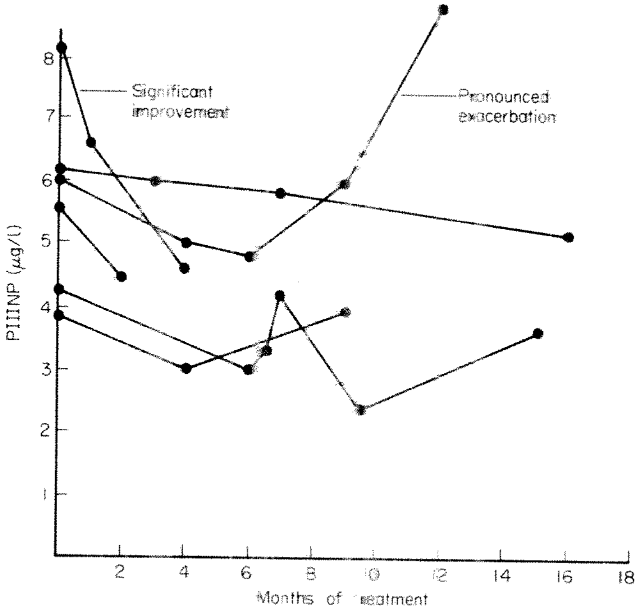

FIGURE 1. Serial PIIINP levels in six patients with severe SS treated with cyclosporin A.

died in acute renal failure, but it is not clear whether this was due to treatment or to progression of the disease. The case for using CyA in SS is supported by three other reports.[16-18]

The effectiveness of various drugs in SS has always been difficult to determine.[6] However, the impressive softening of sclerotic tissue as found in two of our patients, together with the biochemical data, suggests that CyA is useful in selected severe cases of SS. Another significant improvement was the complete healing of long-lasting severe painful digital ulcers in a young female with pronounced acrosclerosis. This patient had previously been treated with penicillamine, ketanserin, nifedipine and rheo-macrodex without any effect.

Treatment with CyA is probably indicated for early cases of SS with severe progression. Further controlled and long-term studies are needed. Even with a low-dose regime, kidney biopsies should be performed for the detection of chronic renal changes. The finding in one of our patients in the post-treatment renal biopsy of definite damage and in another of possible related toxicity agrees with the cumulative data of long-term high-dose CyA treatment in inducing nephrotoxicity.[19] However, one drug commonly used in the treatment of SS, penicillamine,[1,2] is also potentially nephrotoxic.

Finally, single-drug immunosuppressive regimes are not generally very effective in treating severe cases of connective tissue disorders. In the future, CyA may be used as a part of a regime using several immunosuppressive drugs for severe SS.

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Development of folliculitis and pyoderma gangrenosum in association with abdominal pain in a patient following treatment with isotretinoin

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SUMMARY

A 19-year-old man with severe acne developed extensive scalp folliculitis and later superficial pyoderma gangrenosum following treatment with isotretinoin. A cyclical neutropenia was noted and bone marrow findings suggested myelodysplasia. We believe that isotretinoin was implicated in the development of overt symptoms in this patient whose haematological condition was previously asymptomatic.

CASE REPORT

History

The patient developed severe acne in March 1985. He was initially treated with systemic antibiotics in adequate doses (minocycline, cephalexin, and vibramycin) without a satisfactory response. His skin deteriorated with the development of painful nodulocystic lesions and he was treated with prednisolone. A routine blood count revealed a thrombocytopenia but investigations did not reveal any underlying cause. A thrombocytopenia which was not investigated had been noted 1 year previously when he was an in-patient for the investigation of a painful hip. He was treated for 4 months with isotretinoin 80 mg daily (1 mg/kg per day) which produced resolution of his acne. Within 2 weeks of stopping isotretinoin he developed episodes of pustular lesions on the scalp and abdominal pain. The patient felt unwell in the attacks but there was no vomiting or diarrhoea. At this time, swabs from the scalp lesions grew *Staphylococcus epidermidis*. The lesions settled with courses of minocycline (50 mg q.d.s), erythromycin (500 q.d.s), and clindamycin (150 mg t.d.s). The abdominal pain tended to resolve after 7 days but the recurrent episodes persisted. However, by 1988 the skin lesions were not responding to antibiotic therapy. The abdominal pain also became more severe and necessitated hospital admission for opiate analgesia.

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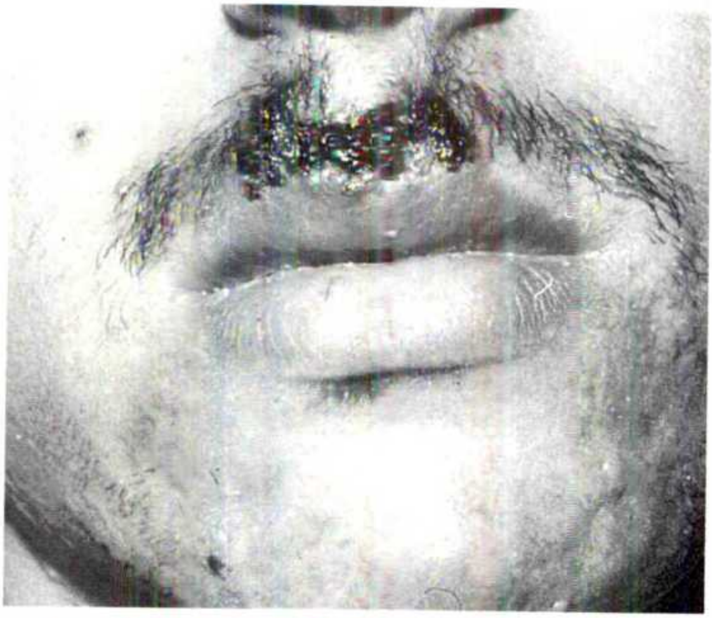

FIGURE 1. The appearance of the lesions during the attacks of folliculitis.

He was referred to Leeds Infirmary for further investigation. The attacks now appeared to occur at monthly intervals. Over 24–48 h pustules would develop on the scalp, neck and beard area which progressed into oedematous, tender fluctuant areas, a number of which became necrotic (Fig. 1). The generalized abdominal pain was associated with some tenderness but with no other signs. In November 1988, the cutaneous episodes became more severe and frequent, now appearing every 2 weeks. The skin lesions changed their character and resembled superficial pyoderma gangrenosum, the lesions occurring predominantly on the trunk (Fig. 2). As the frequency of abdominal pain did not change, the skin and gastrointestinal features occurred more asynchronously.

Investigations

The abnormal investigations were for thrombocytopenia, cyclical neutropenia, and intermittent normochromic normocytic anaemia. The bone marrow was abnormal, showing plentiful dysplastic megakaryocytes and mild trilineage dysplasia. A trephine biopsy showed that the bone marrow cellularity was normal. There was a relative increase in the erythroid series showing a left shift and nuclear atypia. Megakaryocytes were present but sparse and the myeloid series was deficient of more mature forms, with occasional small aggregations of blast cells, suggestive of myelodysplasia. Platelet immunoglobulins were raised. The initial follicular lesions grew *Staphylococcus epidermidis*, one of the two strains of which was haemolytic, and the later lesions were sterile. Histology of the initial pustular lesions showed a folliculitis with a predominantly acute inflammatory infiltrate; but, in addition, there were some changes of vessel damage and fibrin suggesting an accompanying vasculitis. Histology of the latter lesions showed ulceration and vasculitis in keeping with the clinical diagnosis of pyoderma gangrenosum. Antibodies to extracellular enzymes of *S. epidermidis*, *S. haemolyticus* and *Propionibacterium*

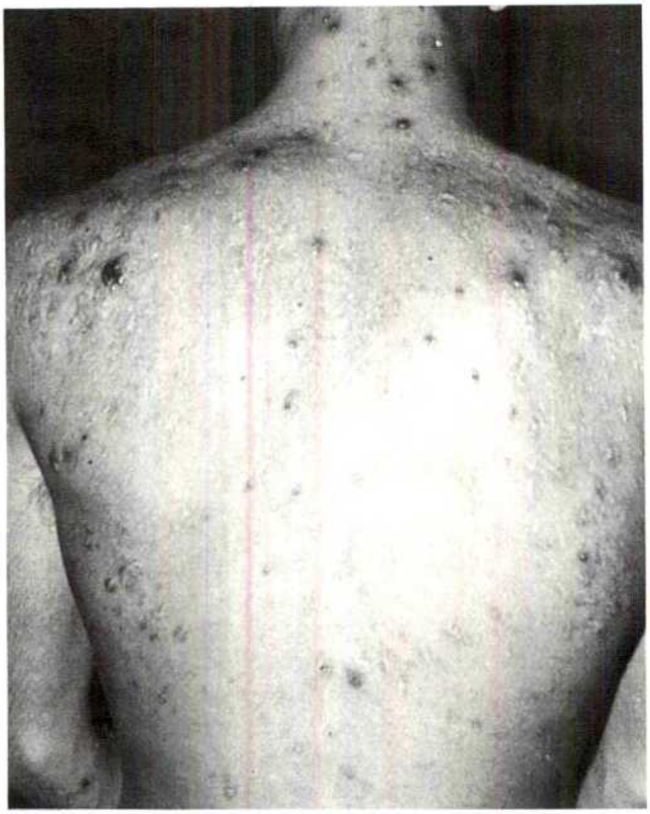

FIGURE 2. Superficial pyoderma occurring on the patient's trunk.

acnes were at the lower limit of normal. The following investigations were normal: urea, liver function, extensive viral screen including HIV antibody, autoantibody screen, immunoglobulins, all white cells except neutrophils, natural killer cells, platelet antibodies, neutrophil and lymphocyte function, and abdominal investigation including barium studies of stomach, small and large bowel, endoscopy, ultrasound, and serum amylase.

Treatment

A diagnosis of moderately severe folliculitis following isotretinoin was initially made and this was treated with appropriate antibiotics (erythromycin, cloxacillin, and clindamycin). This gave some improvement in that it reduced the length and severity of the attacks but did not prevent their recurrence. The superficial pyoderma was intially treated with a potent topical steroid cream (beclomethasone dipropionate 0·025%), which caused resolution of the lesions but did not prevent their recurrence.

A course of oral prednisolone resulted in rapid resolution of both the skin lesions and the abdominal pain. The initial dose was 40 mg and this was reduced to 20 mg by the time of discharge. Over the past 6 months he has required prednisolone at a dosage of 20 mg/day to suppress his symptoms. This treatment has led to a rise in the haemoglobin and neutrophil count but the platelet count has remained depressed.

DISCUSSION

This patient developed moderately severe folliculitis, pyoderma gangrenosum, and abdominal pain in association with thrombocytopenia, cyclical neutropenia and an abnormal bone marrow. This occurred following a course of isotretinoin for his acne.

Folliculitis has been reported following isotretinoin.[1] The most common organism to be isolated is *S. aureus*, although in some patients an organism is not grown. Haemolytic *S. epidermidis* was isolated from the lesions on the scalp of our patient. This organism is well documented as causing pathogenic infection in immunosuppressed patients[2] and we considered that the cyclical neutropenia may have contributed to the attacks of folliculitis.

Both neutropenia and thrombocytopenia have been reported following isotretinoin,[3,4] and a recent report[5] quotes a case of cyclical neutropenia following isotretinoin, although further details of the relationship are not given. Our patient who had thrombocytopenia undoubtedly had a bone marrow abnormality prior to isotretinoin which was asymptomatic. Cyclical neutropenia can be associated with abdominal pain[6] and, although the cause of this is presumed to be sepsis, the precise mechanism is uncertain. The neutrophil count in our patient fell at the time of the cutaneous and abdominal attacks; however, this was to just below the normal range, unlike the unrecordable levels usually documented in hereditary cyclical neutropenia.

Isotretinoin has also been reported to interfere with the function of neutrophils[7,8] and to reduce the numbers of natural killer cells.[9] We could not detect any abnormality in either of these parameters. Acne fulminans has been reported to be associated with vasculitic skin lesions and haematological abnormalities,[10] particularly of neutrophils.[11] Isotretinoin has been reported to have precipitated acne fulminans in one patient.[12] Our patient demonstrated an explosive form of acne rather than acne fulminans because of the lack of ulcerative lesions, systemic symptoms and response of his acne to isotretinoin. The patient has no associated cause of pyoderma gangrenosum other than the myelodysplastic syndrome.

Isotretinoin has effects on the bone marrow, particularly neutrophils, and perhaps this should be taken into consideration prior to treatment. Our patient had similar changes in the bone marrow prior to treatment with isotretinoin but this was asymptomatic. Though retinoids have been advocated for the treatment of myelodysplastic syndrome,[13] this present report suggests caution in their use for this condition.

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Keratitis, ichthyosis and deafness (KID)-syndrome: report of three cases and a review of the literature

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SUMMARY

We report three patients with keratitis, ichthyosis and deafness (KID)-syndrome. All had characteristic hyperkeratotic skin lesions and neurosensory hearing defects. Two had ophthalmologic symptoms. The third patient did not have eye involvement at the age of 3 years, but exhibited the other typical signs of the syndrome. In none of the three cases were any of the patients' relatives affected, and a spontaneous new mutation is the most likely explanation for the occurrence of this rare syndrome. Histopathological and electron microscopic studies revealed orthohyperkeratosis but no other pathology and no abnormal deposits of glycogen were found. Treatment with the aromatic retinoid etretinate proved to be of little value in any of the patients. The necessity for early audiologic and ophthalmologic evaluation and the need for life-long medical care for patients with KID-syndrome is emphasized.

A distinct genodermatosis, the association of hyperkeratotic skin lesions, a neurosensory hearing defect, and a vascularizing keratitis occurring in early infancy or childhood, has been termed the keratitis, ichthyosis and deafness (KID)-syndrome by Skinner *et al.*[1] The earliest publication of a patient with features of this syndrome dates back to 1915.[2] Here, three patients with KID-syndrome are described, who have been seen in the Departments of Dermatology of Vienna and Innsbruck, Austria.

CASE REPORTS

Case 1

N.M., male aged 8. The boy was born after an uncomplicated pregnancy. The family history was unrevealing, and his sister was normal. Consanguinity was not reported. At birth, his skin was red all over but without scaling. Within the first 5 months, brown–black scaly lesions with deep furrows appeared over the elbows, extensor aspects of the knees and the dorsa of the hands. The skin of the face was red with fine telangiectases and appeared thickened (Fig. 1). The skin

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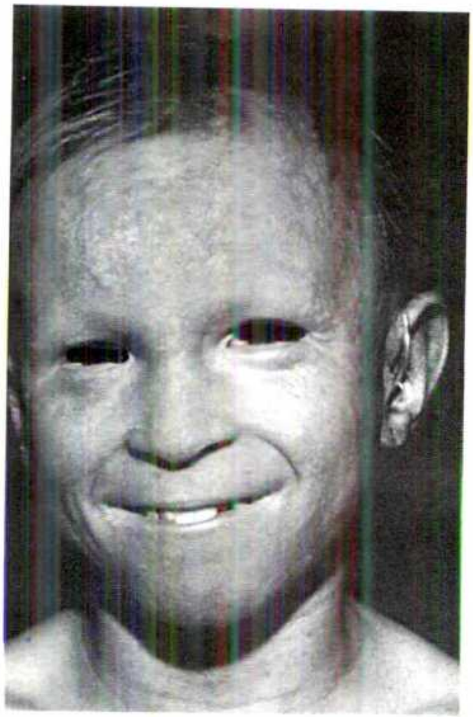

FIGURE 1. Case 1, boy, 8 years old. Scarring alopecia, brownish-red hyperkeratotic lesions on the earlobes and the cheeks; multiple teleangiectases. Note hearing aid.

texture was generally velvety, but in areas was rough and sandpaper-like. On both ears and cheeks greyish-brown hyperkeratotic verrucous plaques were seen. Tiny horny spikes were seen in a follicular arrangement all over the body (Fig. 2). The palms and soles were hyperkeratotic and since the papillary skin lines were both prominent and interrupted, the surface of the skin had a reticulate appearance. There was scarring alopecia on the occiput (due to recurrent

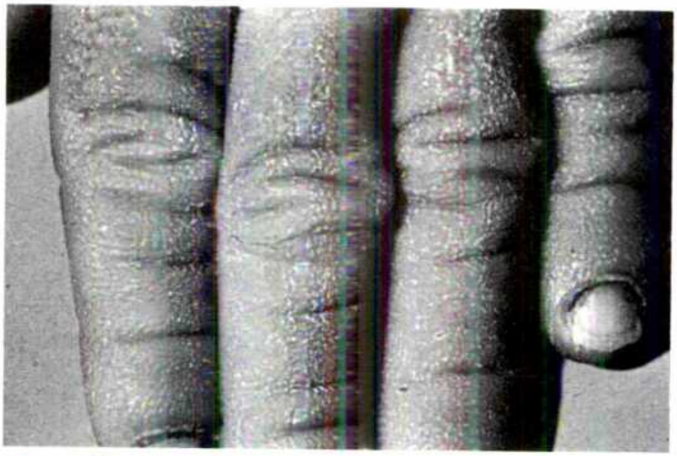

FIGURE 2. Case 1. Tiny horny spikes on the extensor aspects of the fingers.

pyoderma) and on the remainder of the scalp the hair was sparse, short and fluffy and of the vellous type.

White and red blood cell counts, blood chemistry and protein electrophoresis showed no abnormalities. Serum IgE level was elevated (650 kU/l, normal 100 kU/l). Lymphocyte stimulation tests with concanavalin A and phytohaemagglutinin were normal. The excretion pattern of amino acids in the urine was normal; phytanic acid could not be detected in the serum. X-rays of the chest, skull, nasal sinuses, the spinal column and hands showed no abnormalities. Ophthalmologic examination revealed conjunctivitis; the corneas, lenses and fundi were normal as was his visual acuity. A bilateral neurosensory hearing defect had been diagnosed at the age of 4, and the boy had bilateral hearing aids. His mental development was normal.

Treatment with etretinate was started at a dose of 1 mg/kg body weight per day with topical emollients added. After 4 months of etretinate therapy there were no side-effects except for dryness of the lips, but there was no beneficial effect and treatment was switched to 13-*cis* retinoic acid (Roaccutane®), at a dose of 1 mg/kg body weight per day. Severe headaches developed and therapy was changed back to etretinate and moderate improvement was noted, but the treatment had to be stopped again because of an elevation of the serum bilirubin level. A severe relapse occurred within 3 weeks. After this, the patient was lost to follow-up.

Case 2

H.K., female aged 3. The girl was born after an uncomplicated pregnancy. The family history was unremarkable and there was no consanguinity. The patient's 5-year-old sister was normal. The patient's skin at birth was reported to have had a peculiar bluish-white colour and shortly after birth she developed hyperkeratotic lesions. The finger- and toe-nails were absent at birth, but developed during the first 3 years of life, and the toe-nails became dystrophic. At the time of examination, sharply demarcated brownish-red plaques with a velvety surface were present

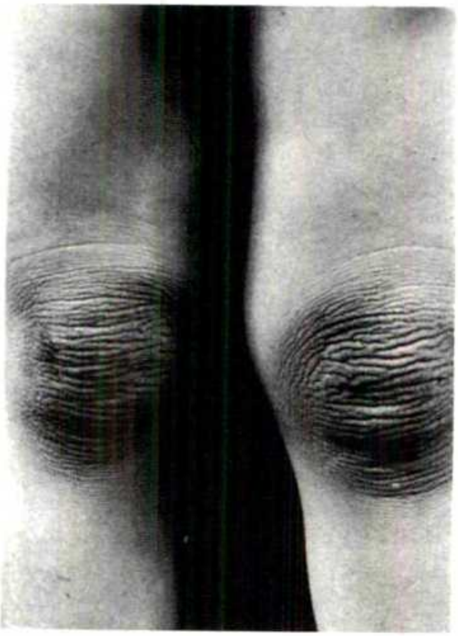

FIGURE 3. Case 2, girl, 3 years old. Brownish, rippled leather-like plaques over the knees.

around the mouth, on the cheeks, the tip of the nose, the chin and the ear lobes. Brownish-grey, hyperkeratotic leather-like plaques with a rippled surface were seen over the knees, the buttocks and the dorsa of the feet (Fig. 3). Her ability to sweat decreased but heat intolerance was not reported. The child had sparse, silvery-white, almost unmanageable scalp hair, but the hair shafts showed no abnormalities. Eyebrows and eyelashes were completely absent. The palms and soles appeared as in case 1.

White and red blood cell counts, blood chemistry, serum IgE levels, serum immunoglobulin levels, and lymphocyte stimulation tests were within normal limits. Tone-audiograms and a brain stem audiometry revealed a moderate-degree bilateral, neurosensory hearing defect localized in the cochlea and also a dysfunction of the acoustic nerve. There was a delay in speech development for which therapy was initiated. Ophthalmological examination was normal as was her growth and mental development.

Treatment with emollients and keratolytic agents (10% urea) did not lead to any noticeable improvement. Etretinate (Tigason®) therapy was initiated at a dose of 0·8 mg/kg body weight on alternate days, and later daily. She has received etretinate elsewhere over a period of 8 months, but without much improvement and this therapy has now been discontinued.

Case 3

G.K., female aged 21. The girl was born after an uneventful pregnancy with normal appearing skin. The family history was unremarkable without any consanguinity. At the age of 6 weeks moist lesions developed on both earlobes and spread over the whole body, but eventually a universal dryness of the skin developed. In the first 2 years of life, the child suffered from multiple recurrent pyodermas. At the age of 5, tinea capitis led to a scarring alopecia and

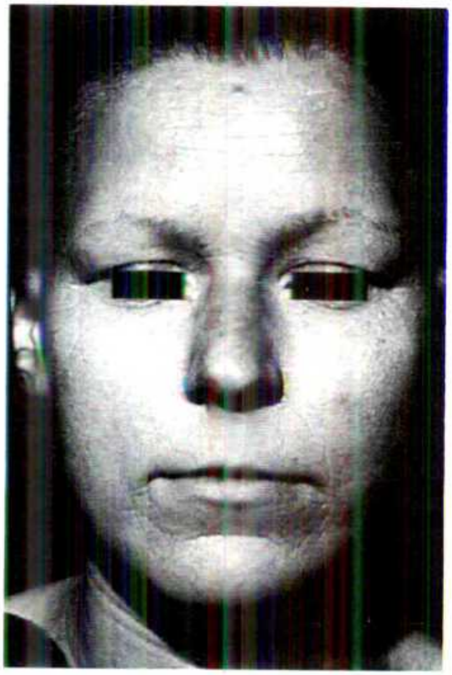

FIGURE 4. Case 3, female, 21 years old. Typical appearance of the face with lichenification of the skin, furrows around the mouth, sparse temporal hair. Numerous milia in the periorbital region.

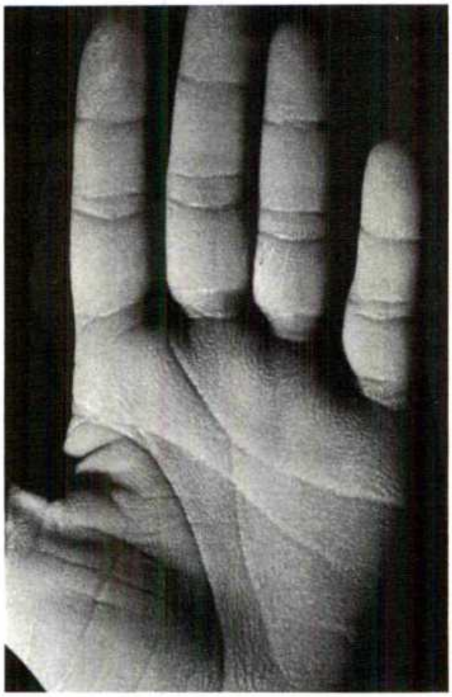

FIGURE 5. Case 3. Peculiar pattern of dermatoglyphics, typical of KID-syndrome patients.

recurrent episodes of conjunctivitis were reported. The whole skin showed diffuse, fine, scaly hyperkeratosis. On the trunk she was anhidrotic, but she sweated normally in the axillae. On examination there were flat erythematous, hyperkeratotic lesions with a velvety to spiny surface appearing symmetrically on both cheeks (Fig. 4). Over the elbows and knees the skin was thickened, greyish, partly red and rippled. The palms and soles appeared as described for case 1 (Fig. 5). The scalp hair was sparse, partly as a result of cicatricial alopecia; the pubic and axillary hair were absent, but no evidence of scarring or atrophy was noticed in these regions. The toenails were dystrophic and the right mammary gland was hypoplastic.

Ophthalmological examination revealed stippled opacities of the corneas, but visual acuity was normal. A high-grade bilateral neurosensory hearing defect had been diagnosed at the preschool age and the patient used a hearing aid. Her intellectual development was normal. White and red blood counts and protein electrophoresis showed normal results. Antinuclear antibodies and autoantibodies against smooth muscle cells, thyroglobulin, thyroid microsomes and gastric parietal cells were not detected. The T-helper/T-suppressor cell ratio was normal. The serum IgE was elevated (980 kU/l; normal range, 100 kU/l).

A trial of PUVA-therapy was discontinued after 3 weeks because of uncontrollable photosensitivity reactions even at low doses of UVA. Retinoid therapy with etretinate at a dose of 1 mg/kg body weight in conjunction with topical emollients led to some improvement but this had to be stopped after 4 years because of intractable pruritus and hair loss.

Histopathology

Skin biopsy specimens from the three patients were taken from various sites (case 1, left knee; case 2, buttock; case 3, left lower leg and left palm) and showed similar features. The epidermis

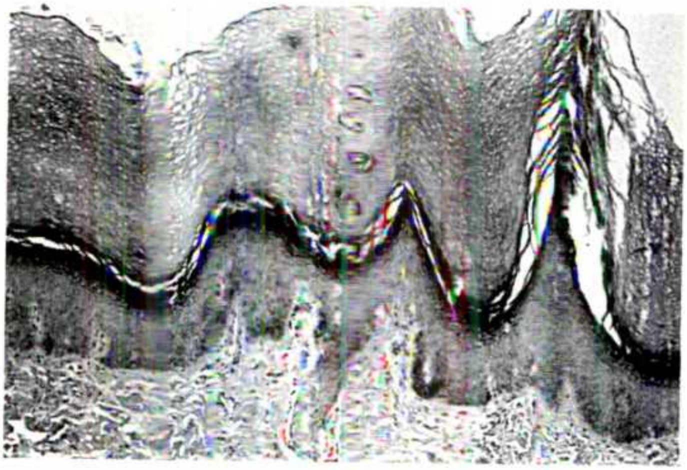

FIGURE 6. Massive hyperkeratosis, hypergranulosis and acanthosis. Sparse lymphohistiocytic infiltrate in the upper dermis (Case 3, left lower leg, haematoxylin and eosin stain, × 200).

was acanthotic with an irregular, spiked surface, hypergranulosis and massive orthohyperkeratosis. The follicular openings were dilated and filled with horny material. The intraepidermal sweat gland ducts were dilated, but otherwise normal. A sparse lymphohistiocytic infiltrate was found in the upper dermis (Fig. 6). The eccrine sweat glands and dermal nerves appeared normal and the PAS-stain did not reveal any abnormal deposits of glycogen. In the specimen from the palm, intraepidermal sweat-gland ducts were found predominantly in the 'valleys' between the horny spikes, where the stratum corneum cells appeared less tightly packed, and therefore less coherent. A second biopsy in case 1 after 10 months of etretinate treatment showed no significant changes.

Electron microscopy

Ultrastructural studies of all three patients showed similar features. The epidermis was acanthotic and normally stratified and the stratum corneum was composed of tightly packed layers of cornified cells. The keratinocytes of the basal and lower spinous layers had abundant cytoplasm with an increased number of organelles. The keratin filament bundles appeared thickened and clumped, and their insertion into desmosomes appeared accentuated (Fig. 7). The membrane coating granules appeared normal and were present in normal numbers. In the stratum granulosum, the keratohyalin granules and inserting keratin filament bundles surrounded the nuclei in a shell-like fashion. Some of the nuclei of the keratinocytes in the upper layers of the epidermis were deeply indented.

Melanocytes, Langerhans cells, the dermal appendages, blood vessels and nerves showed no abnormalities. No abnormal deposits of glycogen or any other material were found.

DISCUSSION

Ichthyosiform skin disorders constitute a large group of genodermatoses which can be classified by their mode of inheritance, clinical appearance, histopathology and associated symptoms.[3] In some, distinctive biochemical defects have been described.[4] Congenital deafness, disorders of hair, nails and teeth and mental retardation are found in a variety of mostly rare syndromes.[5]

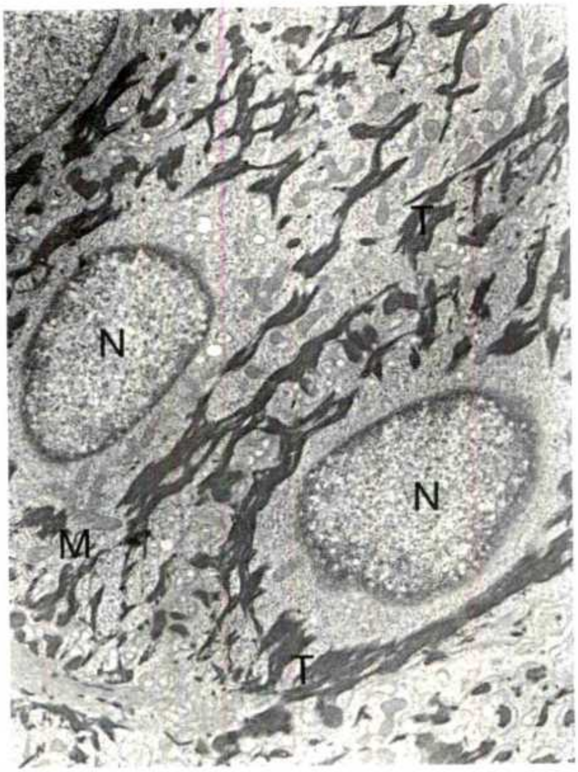

FIGURE 7. Electron micrograph: stratum spinosum. Keratinocytes with abundant cytoplasm, increased numbers of mitochondria (M) and prominent bundles of tonofilaments (T). N, nucleus (Case 1, left knee, × 3000).

The combination of an ichthyosiform dermatosis with deafness and keratitis reported here was first described in 1915[2] and similar cases were reported under various names.[6–16] In 1981 Skinner *et al.*[1] proposed the name keratitis, ichthyosis and deafness (KID)-syndrome for this disorder.

There are a number of clinical criteria for the diagnosis of KID-syndrome. A striking characteristic of patients with KID-syndrome is their close likeness. Children with KID-syndrome look prematurely aged. The skin of the face is dry, with a greyish-brown tone. On the cheeks, the chin and the earlobes are found sharply demarcated, usually erythematous hyperkeratotic plaques. A prominent fold of the lower eyelids is occasionally seen. Hyperkeratotic plaques are typically found over the elbows, knees, dorsa of hands and feet. A fine lamellar hyperkeratosis and tiny horny spikes occur all over the body, and a hyperkeratosis of the palms and soles with a leather-like appearance, tiny pits, spikes and interrupted creases are also characteristic. Other typical signs of KID-syndrome are the sparse and unmanageable scalp hair, the sparse and often absent eyebrows, eyelashes, axillary and pubic hair and often hyperkeratotic and dystrophic nails. In two of our patients only the toe-nails were dystrophic, whereas the finger-nails were normal. The teeth are normally developed in some patients, but in others they have been described as small and malformed, and are likely to develop caries.[1,15–17]

Recurrent skin infections are a major problem in patients with KID-syndrome.[10,12,13,17] Pyodermas, fungal infections often leading to scarring alopecia and recurrent scabies[17] have been reported. A large variety of pathogenic bacteria have been isolated from the skin of patients with this syndrome[1,13,17] but apart from a defect of neutrophil chemotaxis observed in a patient with probable KID-syndrome[18] the propensity of these patients to develop infections remains unexplained.

The definition of the syndrome comprises keratitis and deafness in addition to the ichthyotic skin lesions. Neurosensory deafness is usually present at birth but is often only noted later on in life because speech development is delayed.[1,7-10,13,14,17,19] Each of our patients had a neurosensory hearing defect.

A vascularizing keratitis preceeded by a purulent blepharoconjunctivitis, and leading to corneal ulcerations and pannus formation eventually resulting in blindness, is found in most patients with KID-syndrome. Two of our patients (cases 1 and 3) had recurrent inflammations of the conjunctivae. In one case (3), stippled opacities of the corneae were also found, but these did not impair visual acuity. As the development of eye disorders has been reported to occur as late as the age of 11,[1,6,10,11,15,16,20] these may still develop in our 3-year-old patient, who at the time of examination had no ocular problems. Only a few of the KID-syndrome patients described in the literature were mentally retarded;[21-22] in our patients intellectual development was normal. A unilateral hypoplasia of the mammary gland, which was found in one of our patients (case 3) has occurred in another case.[9] A peripheral neuropathy was not detected in our patients.

Histopathological studies in our patients showed no abnormal amounts of keratin in the sweat-gland ducts. The appendages were normal and contained no abnormal deposits of glycogen as reported in one patient.[20] Electron microscopy showed a normally stratified epidermis with a thick lamellar hyperkeratosis in all three cases, keratin filament bundles were thickened and clumped and the keratohyalin granules were distributed in a shell-like fashion around the nucleus. Peculiarly shaped nuclei with deep indentations were found in all patients, but the significance of this remains unclear. In cultured keratinocytes from a patient with a condition resembling KID-syndrome, no abnormalities of keratinization were found, and immunofluorescence studies of skin biopsies using monoclonal antibodies against prekeratin, filaggrin and involucrin have revealed normal staining patterns.[23]

None of our patients had relatives with skin disorders, hearing impairment or ophthalmologic problems and there was no consanguinity. KID-syndrome occurs mostly as a single case and in both sexes, and may therefore be considered as a result of spontaneous new mutation. However, one example of a vertical transmission of KID-syndrome from father to daughter has recently been reported.[17] An eventually fatal squamous cell carcinoma of the skin developed in a member of this family (father), and squamous cell carcinomas of the skin and the tongue as well as other malignant tumours have been reported in other patients.[21,22,24]

Treatment of patients with KID-syndrome is difficult and only symptomatic relief can be achieved by keratolytic agents and therapy for skin infections. Retinoids have been reported to be beneficial in hyperkeratotic, ichthyotic disorders,[25,26] but as in our cases, seem to be at most of only moderate value for the treatment of KID-syndrome.

KID-syndrome is a rare disorder. Its early diagnosis is important, and the early wearing of hearing aids and starting of speech therapy can prevent the impairment of speech development. Life-long ophthalmologic examinations are necessary to avoid corneal damage. The prognosis seems to be good in general, but no long-term studies of the outcome of KID-syndrome patients exist. The occurrence of malignant tumours in some patients with KID-syndrome suggests that the genetic defect enhances the chance for the development of malignancy, and also emphasizes the need for periodical examination of the affected individuals.

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Cartilaginous pseudocyst of the external auricle in children with atopic eczema

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SUMMARY

Four children with severe atopic eczema developed painless endochondral pseudocysts of the external auricle. The lesions were bilateral in two cases. In one case the lesion spontaneously resolved; of the remaining five lesions, one contained haematoma and the other four contained serous fluid. The aetiology of these lesions and their association with atopic eczema are unclear, but repeated minor trauma from rubbing may play a part.

Of 600 children with atopic eczema referred to our department between 1980 and 1989, four developed cystic swellings of the pinna. Standard textbooks of dermatology[1–3] do not mention this troublesome complication, and we therefore report details of these patients.

CASE REPORTS

Case 1

This boy developed atopic eczema at the age of 18 months. He was referred at the age of 3 years, having had four admissions to hospital for severe eczema which was poorly responsive to topical steroids and emollients: 95% of his skin surface was affected by severely inflamed atopic eczema. His serum IgE concentration was 164,000 IU/ml (normal for age < 42 IU/ml).

At the age of five years he presented with a 2-cm diameter painless fluctuant swelling of the upper anterior portion of the right external auricle which had been present for 2 weeks. It had appeared over a few days. He was apyrexial, and there were no signs of skin infection. The lesion was thought to be an abscess, and it was explored as an emergency procedure. Clear fluid was drained from an intracartilaginous pseudocyst. The incision quickly healed, leaving generalized thickening of the upper anterior part of the auricle with distortion of the contour of the rim, particularly at the upper pole. At the age of 8, the deformed appearance of the ear was unchanged.

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Case 2

This boy developed atopic eczema at 3 years of age. His skin lesions were poorly responsive to conventional topical steroids, emollients and various elimination diets. He was referred at the age of 5 years, by which time he was on prednisolone 25 mg daily. Atopic eczema affected 15% of his skin surface, and was particularly severe on the face. His serum IgE concentration was 6200 IU/ml (normal for age < 52 IU/ml). Prednisolone therapy was discontinued and his eczema progressively deteriorated despite topical therapy with flurandrenolone 0·0125% and clioquinol 3% ointment. By the age of 5·5 years he had severely inflamed eczematous lesions that affected 50% of his skin surface. He was admitted to hospital for exclusive feeding with an elemental diet, which was associated with some reduction in the degree of erythema of the eczematous lesions, and foods were re-introduced singly to identify food triggers.

While in hospital, he was found one morning to have a 2-cm diameter painless cystic swelling of the upper anterior portion of the left external auricle (Fig. 1). At operation 10 days later, it was found that the cartilage of the ear had been split into two layers with a haematoma in the space between the layers. A subperichondral resection of the area was performed, removing all affected cartilage. The histology of the resected cartilage was normal and showed no atypical or degenerative changes. Seven weeks after the appearance of the first cyst, he developed a 1-cm diameter cystic swelling of the upper anterior portion of the right external auricle. At that time, he had active eczema on his ears. His mother commented that he rubbed his ears frequently, particularly during sleep. The swelling enlarged, and over the next 4 weeks became tense, tender and dark in colour. It was explored, and a collection of serous fluid was found between two layers of cartilage. A subperichondral excision was performed, and histology again revealed normal cartilage.

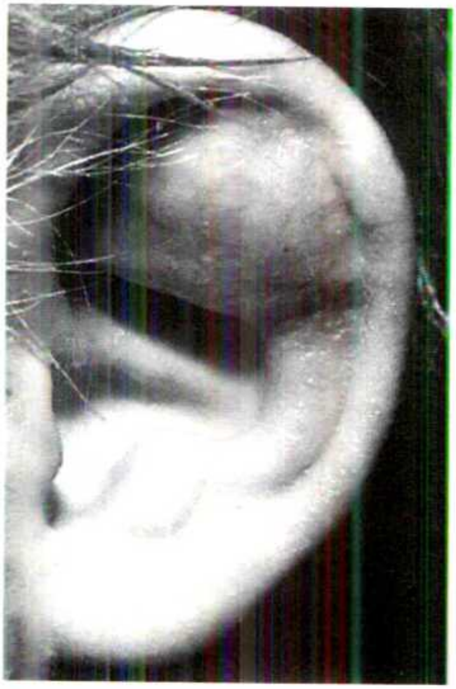

FIGURE 1. Case 2. Cystic swelling of upper anterior portion of left external auricle.

At review 2·5 years after operation, his external auricles were soft and of normal thickness, with minimal distortion of the rim of the right auricle and slight loss of the left antihelical fold.

Case 3

This girl developed atopic eczema at 12 months. She was managed with topical steroids and emollients, and was referred at the age of 3 years. By this time eczematous lesions were present on the limbs, face and neck, and affected 20% of her skin surface. The seum IgE was not measured. There was a history of worsening of the eczema after eating chocolate, and she was tried on two elimination diets without success. She was treated with topical hydrocortisone 1% and clioquinol 3% ointment.

Her eczema persisted, and at 4 years of age she presented with a 1·5-cm diameter fluctuant and painless swelling of the upper anterior portion of the left external auricle, occupying the whole of the scaphoid fossa. No treatment was given, and the swelling resolved spontaneously. Follow up at the age of 6 years revealed an almost normal auricle with only slight thickening of the central part and flattening of the helical rim.

Case 4

This girl developed atopic eczema at 2 months of age. By the age of 6 months 70% of her skin surface was affected. She was treated with topical steroids and emollients and a diet avoiding cow's milk and eggs. At the age of 5 years atopic eczema affected 90% of her skin surface, including her face, and she was admitted to hospital. Her serum IgE concentration was 67,000 IU/ml (normal for age <52 IU/ml). She was treated with an elemental diet with some improvement. At 6 years old her eczema improved dramatically.

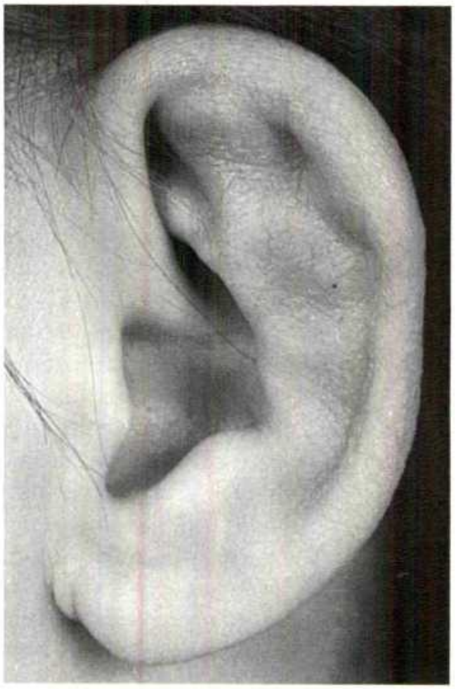

FIGURE 2. Case 4. Irregular cystic swelling of upper anterior portion of left external auricle.

At the age of 6 years 2 months she presented with a painless irregular, fluctuant purple-coloured swelling of the upper anterior portion of the left external auricle (Fig. 2). Over the next four weeks this became firmer and tender, and a 2-cm diameter painless fluctuant swelling appeared on the upper anterior part of the right external auricle. Four months after the appearance of the first ear swelling, her external auricles were explored. The cartilage of the left auricle had been split into two layers, and the space between them contained serous fluid. The perichondrium was abnormally adherent to the affected cartilage over the swelling, and calcification was present at the upper pole. A similar but less extensive lesion was present in the right auricle. The abnormal cartilage was excised, and histology revealed bands of hyaline cartilage surrounded by fibrous tissue but no inflammatory response.

DISCUSSION

We have described four children with severe atopic eczema who developed painless cystic swellings of the external auricle. In two cases these were bilateral, and the second lesion developed 4–7 weeks after the first. In the five lesions which were explored, the auricular cartilage was found to be split into two layers. The space within the cartilage was not epithelialized, and was filled with a clear serous fluid in four lesions and haematoma in one. In the two cases (four ears) where histology was performed, the cartilage showed no inflammation or degeneration.

The nature of these lesions is obscure. In appearance and location they resemble endochondral pseudocysts[4-17] (also known as cystic chondromalacia[12] or seroma of the auricle.[18]) These are intracartilaginous accumulations of serous fluid, occurring most often in young, healthy adult males. Histology shows that the cystic space does not have a true lining, hence the designation of pseudocyst. There is usually no history of major trauma although, in one study, in nine out of 20 patients there was a history compatible with repeated minor trauma such as carrying hampers or large sacks on the shoulder, or lying on a hard pillow.[10] In another case the lesion developed in a man 1 week after he had been 'slapped rather firmly' on the ear by his 4-year-old son.[14] Most cases are unilateral, but bilateral cases have been described.[8,12,15,16] Co-existing skin disease has only been noted once: a 28-year-old man who had severe eczema of the face.[7]

Direct trauma is a well-known cause of an acute auricular haematoma. Such haematomas appear to be particularly common in wrestlers,[19-21] but can occur in other sports such as rugby or boxing. It is said that other types of pressure on the ear, such as lifting upright pianos or large sections of beef, may also cause auricular haematomas leading, if untreated, to chronic thickening and known as 'cauliflower ears'.[22] These haematomas only form on the anterior surface of the auricle, apparently because of the close adherence of the skin to the perichondrium and cartilage.[20] However, there is disagreement as to whether these haematomas form in the plane between the perichondrium and the cartilage or form within the cartilage itself.[19-21] Experiments in which weights were repeatedly dropped onto the ears of rabbits resulted in haematomas which were mostly intracartilaginous,[23] leading to an irregularly thickened ear said to resemble human cauliflower ear. However, subsequent experiments in which blood was injected beneath the perichondrium of rabbit ears demonstrated that this too led to thickening and buckling of the cartilage reminiscent of changes seen in human cauliflower ears.[24]

In our patients there was no history of major trauma. One child had been observed to rub his ears frequently although, as all four patients had eczema on the ears, it is probable that they all rubbed or scratched their ears to some degree. Whether this constitutes sufficient trauma to cause these lesions is unknown. In 1935, Howard described a man with nodular thickening of the ear and reported that, as a boy, this patient had been in the habit of fingering or 'foxing' his ear.[22] Howard stated, without any supporting evidence, that trauma to the auricle could cause either a subperichondral serous effusion or a haematoma, and suggested that the two were related entities.

All our patients had been treated with elimination diets, raising the possibility that malnutrition[25] may have somehow caused or predisposed to these lesions. However, all these diets were closely supervised by a dietitian, to ensure adequate intakes of protein, energy, vitamins and minerals. It is likely that the dietary restriction in these patients is a reflection of the severity of the eczema and its lack of response to topical treatment, rather than the cause of the lesions.

The aetiology of these endochondral pseudocysts and the reason for their association with atopic eczema remain obscure. Trauma from scratching and rubbing is a possible explanation. Spontaneous resolution occurred in one patient and, based on our experience, it would seem reasonable to defer surgery and reserve it for lesions which fail to resolve, although it is technically more difficult to excise the swellings after many months when the fluid is largely replaced by fibrous tissue. Simple incision and drainage, performed in our first patient, gave a very poor cosmetic result, and resection of the affected cartilage with preservation of the perichondrium would appear to be the best approach.

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Cowpox presenting with sporotrichoid spread: a case report

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SUMMARY

We describe a patient with cowpox infection who presented with a sporotrichoid pattern of cutaneous lesions. This is the first report of a sporotrichoid pattern of spread in cowpox infection.

Cowpox holds an important place in the history of medicine. For many years cowpox was used to confer immunity to smallpox. Following the eradication of smallpox, prophylactic vaccination is no longer required, and natural cases of cowpox infection in humans have been increasingly recognized.

CASE REPORT

An 18-year-old student presented with a 3-week history of swelling of the left cheek and a stye-like lesion on the left upper eyelid. The onset of the swelling had been accompanied by an influenza-like illness, and the condition was unresponsive to broad-spectrum antibiotics. He had previously spent a month working in the garden of the new family home built on reclaimed farmland. He owned a pet adult male cat which occasionally scratched him, but had no other contact with animals.

On examination he was found to have ulcerated nodules on the left upper and lower eyelids, and numerous deep nodules extending in a linear sporotrichoid pattern across the left cheek (Fig. 1). The left preauricular and cervical lymph nodes were enlarged.

Investigations

Full blood count, serum biochemistry and glucose were normal. Microbiological examination of pus from the lesions was negative. A biopsy from one of the ulcerating lesions showed a deep dermal granulomatous infiltrate surrounded by plasma cells, foci of necrosis and an acute inflammatory infiltrate. Stains for fungi, bacteria, acid-fast bacilli and viral inclusions were negative. The diagnosis of cowpox was made when a culture of material from one of the ulcers,

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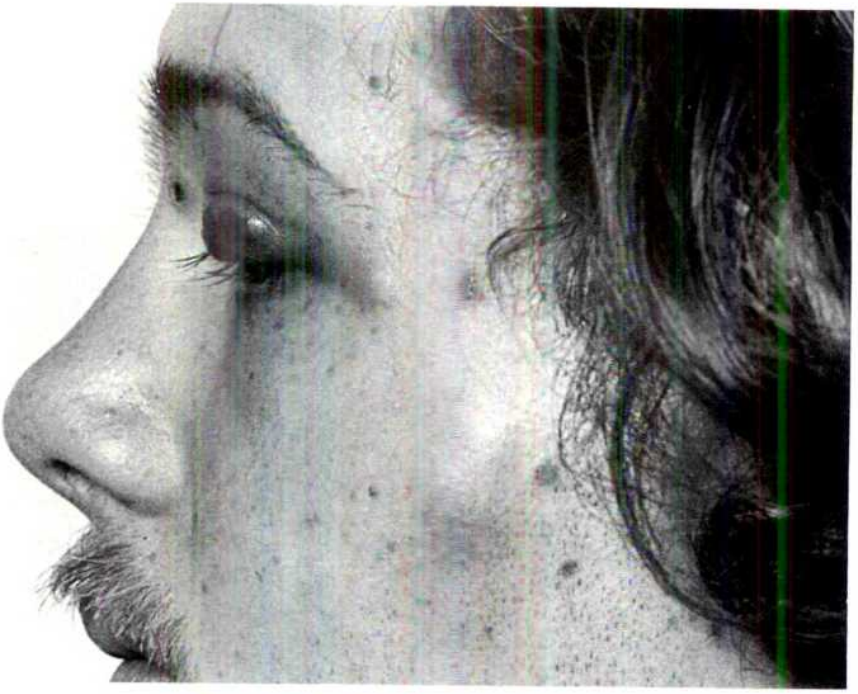

FIGURE 1. Sporotrichoid pattern of cowpox lesions.

on chorio-allantoic membranes of fertile hens' eggs, produced the typical haemorrhagic lesions of cowpox virus infection. Antibodies to orthopox virus were found at a titre of 1 in 512 in convalescent serum from the patient, and at a titre of 1 in 64 in serum from the patient's cat. (The patient had never been vaccinated against smallpox.)

DISCUSSION

A sporotrichoid pattern of infection has not previously been reported with cowpox infection, which usually produces a single ulcer and local lymphadenopathy. A proximal linear spread of infective lesions, presumably along lymphatics, is typical of sporotrichosis, and has been reported in atypical mycobacterial infections, nocardiosis, leishmaniasis and cryptococcosis.[1-4] We initially considered an inoculation mycobacterium infection to be the most likely diagnosis. Cowpox, smallpox and vaccinia are orthopox viruses and share common antigens. Antibodies to one orthopox virus confer protection against the others and this principle has been used in vaccination for many years.

Cowpox holds a unique place in the history of medicine and smallpox.[5] For centuries, smallpox was a severe disease associated with many fatalities in every part of the world. The disease left its survivors pock-marked and it was realized that pock-marked persons never caught smallpox again. Inoculation of susceptible individuals with smallpox pus or scabs produced a much less severe form of the disease and conferred protection from fatal smallpox. Variolation, as this practice became known, was widely practised during the second half of the eighteenth century in Europe, but despite the advantages of variolation it was recognized that occasionally inoculated subjects died or conveyed severe disease to susceptible contacts.

Edward Jenner is credited with recognizing the advantages of the use of cowpox to confer immunity to smallpox. He had been called upon to variolate people in rural communities and found that many resisted his efforts to induce mild smallpox infection. These patients had previously had a disease they called cowpox, contracted by milking cows that had an eruption on their teats. Jenner found, however, that some of those who seemed to have had cowpox nevertheless were susceptible to variolation. From further investigations Jenner was to learn that cows' teats were subject to various eruptions and that these were all capable of communicating sores to the hands of milkers. Furthermore, all sores derived from the animals were loosely called 'the cowpox'. Jenner carefully distinguished the features of 'true' cowpox which conferred immunity to smallpox, from 'pseudo-cowpox' which possessed no such powers. ('Pseudo cowpox' is a bovine orf virus infection which may produce 'milkers' nodules' on the hands of milkers.) He demonstrated that artificial inoculation with cowpox material protected against smallpox. This practice became known as vaccination and replaced variolation.

In 1939 it became possible to characterize the properties of the virus present in the vaccine material. At this time it was discovered that the vaccine virus had unique properties which distinguished it from the virus of natural cowpox, and it was therefore named vaccinia virus.[6] It must be assumed that Jenner's original cowpox became replaced by vaccinia during its artificial propagation.

Despite its name, cowpox is not enzootic in cattle, although bovine cases do occur.[7] It is probable that cowpox virus, which has been demonstrated to have a wide host range, produces enzootic infections in a variety of rodents from which it is occasionally transferred to other animals: cows, cats, zoo animals and sometimes man. In turn, cows, cats and sometimes zoo animals could serve as a source of infection for humans. Attention has been drawn to the domestic cat which has been recognized increasingly as a source of human infection.[8-11] We speculate that the patient's cat became infected with cowpox following contact with an unknown reservoir host, probably a small wild mammal, and subsequently transmitted the condition to the patient by contact.

The discontinuation of routine vaccination against smallpox may be expected to lead to an increase in the population susceptible to orthopox virus infections, and we may expect to see an increase in cases of human cowpox infection which should be considered in patients who live on, or visit rural farms or smallholdings and who may have contracted the infection through contact with a domestic cat.

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Acral granulomatous dermatosis

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SUMMARY

A 17-year-old boy developed an acral granulomatous dermatosis which resembled clinically acrodermatitis continua of Hallopeau. The histology showed dermal and subcutaneous abscess formation with granulomas. The lesions responded to systemic corticosteroid therapy, with residual atrophy and contractures of the fingers.

Acral dermatoses are relatively uncommon and many cases are due to non-specific inflammatory conditions such as contact dermatitis, or chronic infections such as paronychia. Rarely, persistent acral pustulation occurs of the type described by Hallopeau as acrodermatitis continua. We describe a distinctive type of acral dermatosis with the formation of granulomas.

CASE REPORT

A 17-year-old boy was admitted in December 1988 with fever, malaise, arthralgia and tender swellings of the fingers. He had been well up to 3 months previously when he developed a fever, as high as 38·0–38·5°C, and a painful swelling at the tip of the left small finger. Despite treatment with various antibiotics, new lesions appeared on other fingers and on his forehead.

On examination, there were erythematous indurated and pustular lesions on the fingers and forehead (Fig. 1). The pustulation of the nail bed and matrix had led to loss of the nail plate and severe onychodystrophy (Fig. 2).

The laboratory findings were haemoglobin 12·1 g/dl, red cell count $450 \times 10^4/\text{mm}^3$; leucocyte count $19{,}200/\text{mm}^3$ with 87% neutrophils; the erythrocyte sedimentation rate was 76 mm in one hour; platelet count $62 \times 10^4/\text{mm}^3$. The liver enzymes were normal except for slightly elevated GPT, 97 IU/l, (normal 4–33 IU/l) and γ GTP, 72 IU/l, (normal 3–62 IU/l). The total serum protein was 7·2 g/dl and electrophoresis showed a decrease in the albumin (47·2%) and an increase in the globulins (α_1: 4·9%, α_2: 16·6%, β: 10·6%, γ: 20·8%). The serum IgG was 1884 mg/dl (normal 780–1450 mg/dl), C3 200 mg/dl (normal 79–135 mg/dl) and fibrinogen 915 mg/dl (normal 200–400 mg/dl). C-reactive protein was 15·3 mg/dl (normal less than 0·3 mg/dl). The

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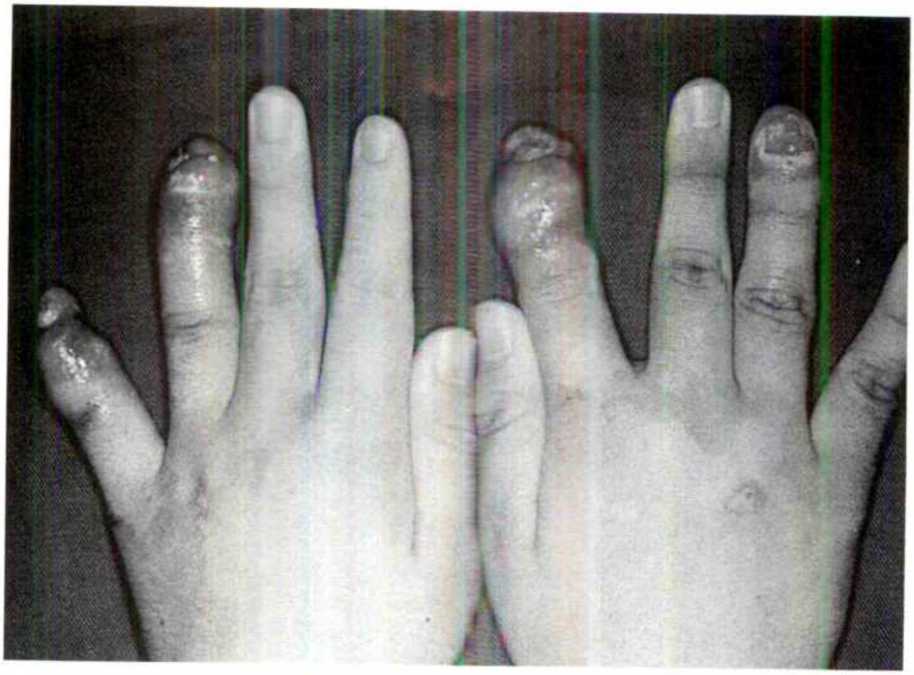

FIGURE 1. Indurated erythematosus lesions on the fingers.

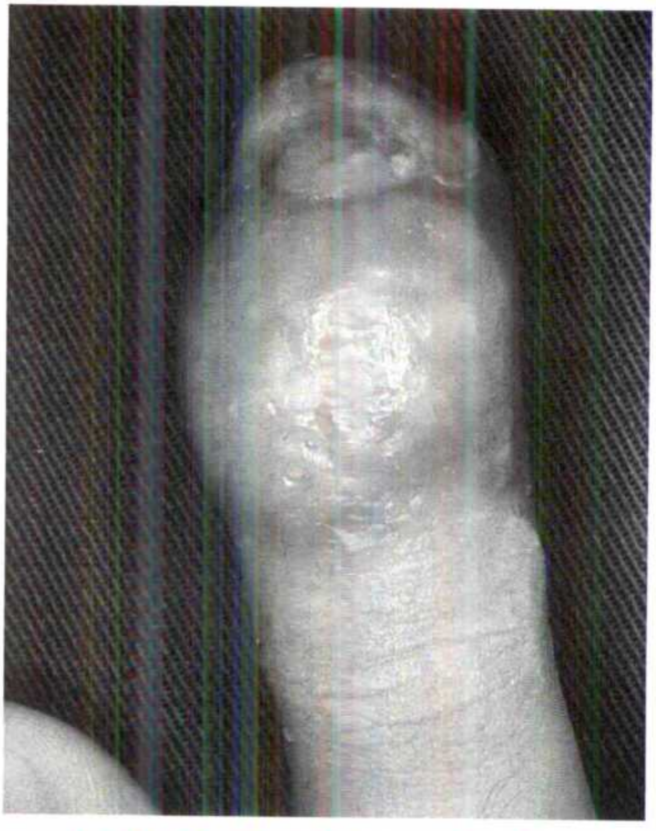

FIGURE 2. Pustular lesions with onychodystrophy of the right index finger.

plasma vitamin B12 was raised, being 3603 pg/ml (normal 300–960 pg/ml) and there was a low level of iron, 17 μg/dl (normal 120–140 μg/dl). The plasma copper was 196 μg/dl (normal 78–131 μg/dl). Normal or negative results included urinalysis; serum electrolytes, blood glucose; urea N; serological test for syphilis; serum levels of IgA, IgM and C4; total haemolytic complement; immune complexes using the C1q binding assay; rheumatoid factor; antinuclear antibodies and anti-DNA tests; anti-ENA antibodies; blood ratios of T and B lymphocytes; and lymphocyte blast transformation assays. No circulating IgG, IgA or IgM antibodies were detected by the indirect immunofluorescence technique using normal human skin as substrate. The chest X-ray and electrocardiogram were normal. X-rays of the sinuses were normal. Human lymphocyte antigen genotypes were A2, Bw46, Bw61, Cw3, DR4.

All four biopsy specimens obtained from early lesions on the fingers and forehead showed, on histology, necrotizing granulomas involving the deep dermis and subcutaneous tissues beneath a normal epidermis (Fig. 3). The granulomas were not necessarily arranged around the follicles and were characterized by areas of necrosis surrounded by an infiltrate that contained neutrophils, lymphocytes, and epithelioid cells, but only rarely eosinophils. Multinucleated

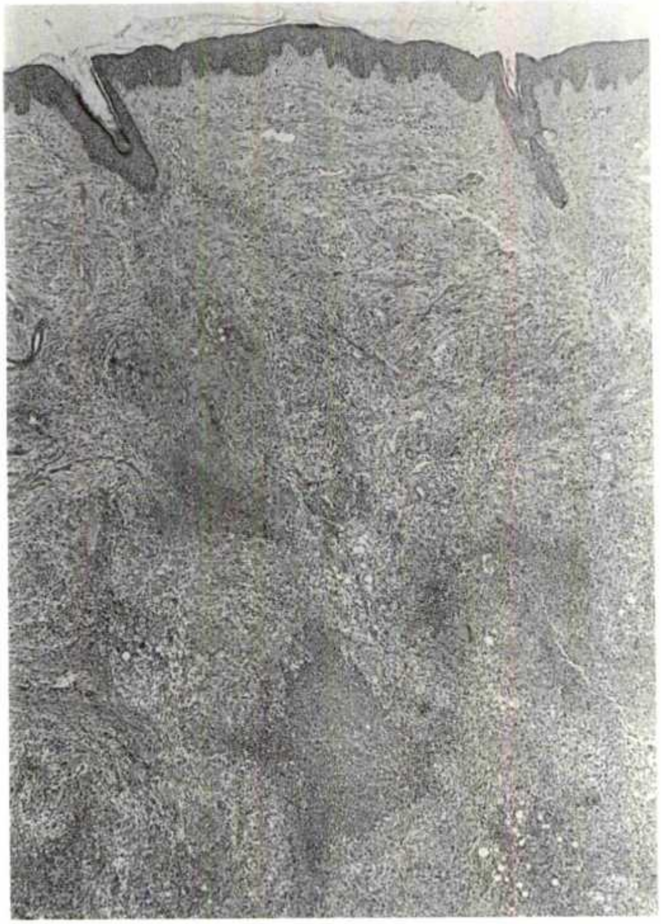

FIGURE 3. Biopsy showing granulomatous inflammation with necrosis in the deep dermis. Haematoxylin and eosin, × 28.

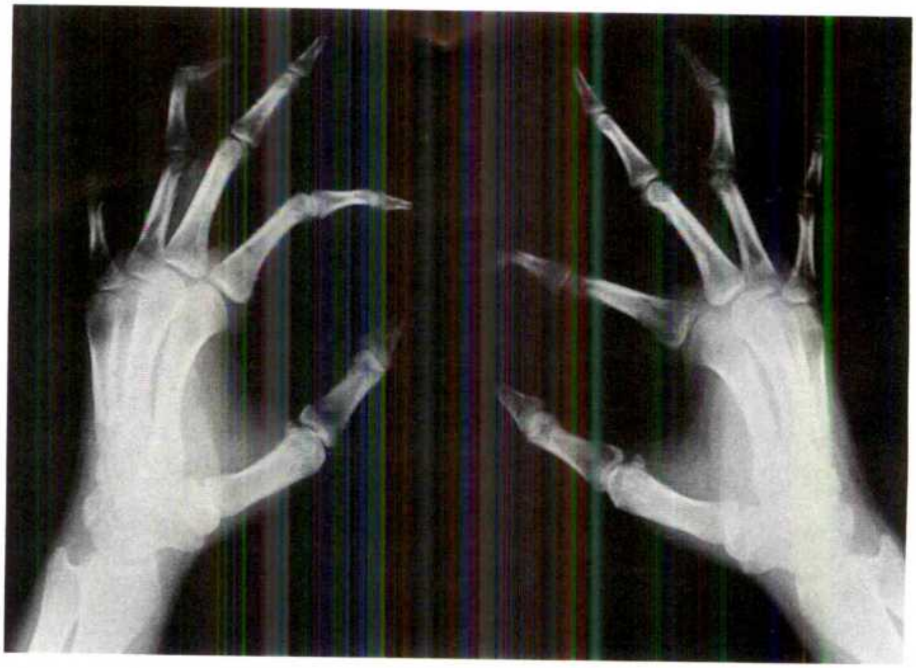

FIGURE 4. X-ray of the hands (February 1989).

giant cells were scattered around the areas of necrosis. Some arterioles showed intimal thickening with endothelial proliferation, but were not infiltrated with inflammatory cells. The elastic laminae of vessels remained intact. Direct immunofluorescence demonstrated the deposition of fibrinogen around the blood vessels in the dermis, but not immunoglobulins or complement. The pustules contained necrotic material from the dermis together with whole and fragmented leucocytes. Special stains for fungi and acid-fast bacilli were negative. Bacterial and mycological cultures were negative.

The patient was treated with etretinate in an initial dose of 30 mg daily, without any improvement. The disease gradually progressed resulting in tissue necrosis and deformity of the fingers. X-rays revealed destruction of the terminal interphalangeal joints with atrophy of the distal phalanges (Fig. 4). Treatment in February 1989 with prednisolone in an initial dose of 40 mg daily produced an improvement. He became afebrile and the cutaneous lesions resolved to leave severe atrophy with contractures of the affected fingers. All the abnormal laboratory investigations returned to normal over the next 4–6 weeks. He has been on 10 mg of prednisolone and 100 mg of azathioprine daily for the past 4 months and continues to be in remission.

DISCUSSION

This case of acral dermatosis could be confused with other similar acral and pustular eruptions. There was no evidence that any micro-organisms played a role in the causation of the disease and cultures and smears were negative for bacteria and fungi. Treatment with various antibiotics was ineffective in our case, and the good response to the systemic corticosteroid therapy suggests that immune mechanisms might be responsible for the tissue damage to the fingers.

A disease that shows a very similar clinical picture is acrodermatitis continua of Hallopeau,[1] in which nail destruction and atrophy of the distal phalanx also occur. Though the condition in our case resembled acrodermatitis continua in this disorder the primary lesion is the formation of pustules in the epidermis.[2] In our patient the epidermis appeared normal. Wegener's granulomatosis usually involves the upper and lower respiratory tracts and is characterized by necrotizing and granulomatous vasculitis, and very rarely occurs on the hands.[3] Pyoderma gangrenosum is distinguished by characteristic ulceration,[4] and recurrent febrile neutrophilic dermatosis of Sweet[5] by painful red papules/nodules accompanied by fever and a neutrophilic-leucocytosis and can be readily distinguished from the distribution and character of the lesions. Though in this disorder there is a dense neutrophil infiltrate, there are no granulomas.

Our patient had evidence of systemic involvement with fever, an elevated erythrocyte sedimentation rate, anaemia, leucocytosis, thrombocytosis, elevated C-reactive protein level with abnormal liver function tests and plasma levels of fibrinogen. These abnormal investigations returned to normal after the patient was started on prednisolone therapy. Whether our case represents a unique and separate entity, or an early stage of a systemic disease remains unresolved.

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Venous stasis ulcers, stasis dermatitis and mothers' political ambitions for their offspring

SIR, At the age of four, following my prescription of nettles and whitewash for an ailing racehorse, my mother determined that I should be a clever doctor. At the risk of being too clever by half, I suggest that J.L.Burton's observations on venous stasis[1] show a lack of understanding of the microcirculatory bed. The capillaries that supply the epidermis are a network which even in healthy tissue show some stops and starts in the flow of the blood that they contain. Observation of the capillary bed by capillary microscopy or the more sophisticated TV microscopy, shows that the stops in the capillary bed are not picked up by the laser Doppler and it is common to have fast flow in slightly deeper shunts when there is no flow in the overlying capillaries. Anyone who has observed the dilated capillaries that characterize the kind of vasculature seen in chronic leg ulceration with livedoid vasculitis will know that when the subject is standing, there can be virtually no flow for minutes in the capillaries that supply the epidermis. It may be because of this that blood diverted through deeper channels that do not supply a metabolically demanding tissue, will still retain oxygen when it reaches the femoral vein. Bollinger *et al.*[2] using fluorescence video microscopy demonstrated late appearance of dye in some capillaries and others received no fluorescent tracer at all. An in depth discussion of this phenomenon was reported[3] in the following terms: 'Fagrell in his paper to the British Microcirculation Society (and as was demonstrated in several of the workshops) was at pains to emphasize that flow in superficial capillaries supplying the skin shows great moment to moment variability, known as vasomotion, and did not always correlate in moment to moment changes in total blood flow, as measured by techniques such as the laser-Doppler flowmeter. Lack of correlation between superficial blood flow and deep mid-dermal or subcutaneous blood supply is a consequence of local factors in the capillary, such as haemoconcentration, white cell rheology and variable shunting within the capillary bed through preferential channels. Fagrell pointed out that redness and pulsation in an arterial bed can be noted at the surface of the skin at the same time as no flow is observed from the most superficial capillaries'.

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Reply

SIR, My friend Terence Ryan, it seems, has now replaced whitewash with hogwash. Since capillary stasis lasting for several minutes can occur even in normal skin,[1] it is not surprising that it can also be observed in the skin around leg ulcers. But capillaries are not veins, and it is totally inappropriate to use the term *venous* stasis for such hyperaemic tissue. Whether *capillary* stasis plays any part in the pathogenesis of venous leg ulcers is very debatable. Partsch[2] showed that the mean transcutaneous oxygen pressure was low in the skin around venous leg ulcers, but the inhalation of pure oxygen for 3 min caused a fivefold increase in the cutaneous oxygen pressure. This is what we should expect if there is a pathological diffusion barrier around the capillaries, but such a rise of oxygen pressure would not occur in the presence of significant stasis.

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Alopecia areata treated with diphencyprone: is an allergic response necessary?

SIR, The contact allergen diphencyprone (DPC) has been used in the treatment of alopecia areata (AA), totalis and universalis, with varying degrees of success.[1,2] It is an effective form of therapy for mild to moderate AA of the scalp, but less effective when the loss is more extensive; although we have personal experience of its success in both alopecia totalis and universalis.

Contact allergens are thought to act by the initiation of a delayed hypersensitivity reaction resulting in a specific T-suppressor-cell response in the upper epidermis and a non-specific T-suppressor-cell response in the dermis around the hair follicles. This influx of T-suppressor cells is thought to redress the imbalance of helper: suppressor cells present in the perifollicular lymphocytic infiltrate found around hair follicles in biopsies taken from patches of active AA.

Following sensitization the optimum concentration of DPC used is thought to be that which stimulates the production of erythema and pruritus in the area of treatment for 36 h after application. A reaction of this type has been considered necessary for successful treatment. However, Botham *et al.*[3] state that presensitized mouse epidermis demonstrated an increase in Langerhans cells following the application of a potent sensitizer, dinitrofluorobenzene, at low concentrations which did not elicit an allergic response. This observation suggested to us the possibility of stimulating hair follicles in AA using low concentrations of DPC which did not result in erythema and pruritus.

Fifteen patients (six male, nine female; mean age 41 years, range 16–63 years) with a mean duration of alopecia of 10 years (range 1–60 years) were treated with DPC as previously described.[4] Ten of the 15 had alopecia universalis, three had alopecia areata confined to the scalp and two had alopecia areata of the scalp with involvement of body hair also. Following sensitization with a 2% solution of DPC, the concentration was titrated to a sufficiently low concentration (0·01–0·0001%) to avoid any erythema or pruritus after the application of DPC. No adverse side-effects were noted. After 20 weeks of treatment, none of the 15 had regrown any hair; no vellus hairs, sparse dystrophic hairs or sparse terminal hairs were noted during the period of treatment. In the previous study[4] where the mean age of those with regrowth was 38 years and the mean duration of alopecia was 21 years, the initial regrowth was seen after 5–18 weeks of treatment. It would appear therefore that the eliciting of an allergic reaction after application of DPC is an integral part of successful treatment resulting in hair growth.

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Interactions between the oral contraceptive pill and antibiotics

SIR, The efficacy of the oral contraceptive pill in patients taking antibiotics remains an unresolved question. No clinical studies exist indicating whether or not the pill failure rate is increased in acne patients receiving combined therapy when compared to those taking an oral contraceptive alone.

We sought to address this problem by interviewing the female acne patients attending our out-patient clinic over a 3-year period. Of these patients, only 160 patients had ever been simultaneously prescribed antibiotics for acne and the oral contraceptive pill. These patients were asked to fill in a questionnaire which was completed satisfactorily by 124 of the patients. We asked the name of the oral contraceptive pill, their antibiotics and the length of time during which they were taking the two preparations simultaneously. They were asked whether they had experienced any side-effects while on the antibiotics, in particular diarrhoea or other gastrointestinal side-effects. They were questioned about pregnancies and whether they had ever become pregnant inadvertantly on the pill/antibiotic combination. The number of women years was calculated for the period that the patients continued on the pill/antibiotic combination. This was then compared to the acceptable pill failure rate quoted by the companies manufacturing a combined low-dose oral contraceptive pill using the χ^2 test.

The numbers of patients taking the various antibiotics were as follows: oxytetracycline, 26; erythromycin, 54; cotrimoxazole, 8; trimethoprim, 1; minocycline, 29; doxycycline, 3; clindamycin, 2; and amoxycillin, 1. The total period of time the patients were on both an oral contraceptive and an antibiotic was 163 women years. Two pregnancies were attributable to the pill/antibiotic combination. Neither of these patients had omitted oral contraceptive pills, or developed diarrhoea or other gastrointestinal side-effects. One patient was on Brevinor (oestrogen 35 mg; norethisterone 500 mg) and was taking long-term minocycline, and the second patient had taken oxytetracycline for 5 days while on Microgynon 30 (oestrogen 30 mg; levonorgestrel 150 mg). This failure rate is significantly increased sixfold when compared to the accepted upper limit of normal failure rate of two per 1000 women years[1] ($P < 0{\cdot}05$).

A number of authors have reported pregnancy in women whilst on combined oral antibiotics and contraceptive steroids.[2–4] This subject has been reviewed extensively by Orme *et al.* in 1986,[5] but these authors were unable to produce evidence to suggest that the combination should be avoided. The Committee on Safety of Medicines have received reports, albeit infrequent, of pill failure due to tetracycline, minocycline, cotrimoxazole, trimethroprim, erythromycin and ampicillin.

We feel the increase in the pill failure rate in our study requires further study. We have attempted to increase our data base, but the number of acne patients who are simultaneously prescribed both therapies is small compared to the number of females who take either the pill as contraception or antibiotics for acne. Despite the limited number of cases, our results show a sixfold risk leading us to advise against the use of oral antibiotics in acne subjects who use the pill as a contraceptive.

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Lithium succinate and seborrhoeic dermatitis: an antifungal mode of action?

SIR, Recent reports have demonstrated that topical application of an 8% lithium succinate ointment is of therapeutic value in the treatment of seborrhoeic dermatitis (SD).[1,2] Many recent studies have implicated *Malassezia furfur* (formerly *Pityrosporum* spp.) in the pathogenesis of SD by demonstrating the clinical efficacy of anti-fungal therapy,[3,4] but Boyle and colleagues reported that lithium succinate had no effect on the growth of seven *M. furfur* isolates when it was tested *in vitro* at concentrations of 1–150 mg/l.[1] It is likely, however, that topical application of 8% lithium succinate ointment will result in local concentrations far in excess of 150 mg/l (approximately 1 mM). Moreover, strains of several species of yeast have been reported to be sensitive to 2–30 mM lithium, which inhibited protein and RNA synthesis.[5] We have therefore investigated the response of *M. furfur* isolates to higher concentrations of lithium succinate.

Twelve strains of *M. furfur* were tested by inoculation on to the surface of a lipid-enriched peptone-glucose medium[6] into which were incorporated dilutions of a filter-sterilized solution of lithium succinate to give final concentrations of 1·5–100 mM. Inocula consisted of 12·5 μl of moderately turbid suspensions (1–5 × 10^5 cfu/ml) of 9-day cultures of various laboratory isolates. For comparative purposes the inhibitory activities of lithium chloride and sodium succinate were also assessed. Minimum inhibitory concentrations (MICs) were defined as the lowest concentrations at which there was either no growth or a barely visible film of growth after 7 days aerobic incubation at 34°C.

Results varied markedly among different morphological groups of *M. furfur*. Large colony strains which predominate on the skin of the upper trunk[7] yielded lithium succinate MICs of 100 mM (one strain) or 50 mM (four strains). Small and medium-sized colony strains, which predominate particularly at sites on the head, were more sensitive, with MICs of 6 mM (four strains) or 3 mM (three strains). Parallel determinations of lithium chloride and sodium succinate MICs indicated that lithium was the active moiety.

These results indicate that the *M. furfur* strains most prevalent on the head, the primary site of SD, are inhibited by lithium succinate at concentrations approximately 100-fold less than those available in an 8% ointment. This provides a possible explanation for the efficacy of lithium succinate in SD. The *in vivo* response of *M. furfur* to lithium succinate is currently being monitored in extended trials to further test this hypothesis.

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Generalized pustular psoriasis in relation to withdrawal of cyclosporin A

SIR, Recently a case was reported of life-threatening psoriasis, following withdrawal of treatment with cyclosporin (CyA).[1] We wish to report two patients with chronic plaque psoriasis who developed severe generalized pustular psoriasis when long-term treatment with CyA was decreased and then stopped.

The first was a 49-year-old female with a history of psoriasis for 25 years and complicated by arthritis. Previous treatments had included PUVA, methotrexate and retinoids and had only resulted in partial, short-term remissions. She was initially treated with a dosage of 10 mg/kg per day of CyA and this resulted in clearance of her psoriasis. After 2 years of treatment with 6 mg/kg per day of CyA the dose was reduced to 3 mg/kg per day over a period of 10 months. The patient developed pustular psoriasis at this lower dose, but a further remission was obtained when the dose was increased to 6 mg/kg per day.

The second patient was a 53-year-old female who had not responded to previous conventional treatment. She was started on treatment with CyA at a dosage of 2·5 mg/kg per day without any improvement, but the dose was increased to 5 mg/kg per day with total clearance of her psoriasis. The dose of CyA was slowly reduced and after being in remission the patient developed severe generalized pustular psoriasis a week after CyA therapy was discontinued.

It is now well established that low doses of CyA (3–5 mg/kg per day) can induce remission in a patient who has severe psoriasis.[2] There are however, a number of side-effects such as nephrotoxicity and hepatotoxicity[3,4] that are dose-dependent. The dose of CyA is usually reduced after a period of remission. In most of our patients, after a reduction in the dose of CyA there has been no serious relapse, but in these two patients when the drug was reduced and stopped, pustular psoriasis developed. This rebound phenomenon may be compared to that seen in patients with psoriasis on prednisolone when a reduction or withdrawal of the systemic steroid has taken place. We suggest that an alternative treatment of the psoriasis should be considered when the dose of CyA is reduced or stopped.

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British Journal of Dermatology (1990) **122**, 720.

Book Review

Current Topics in Contact Dermatitis. Edited by P.J.FROSCH, A.DOOMS-GOOSSENS, J-M.LACHAPELLE, R.J.G.RYCROFT AND R.J.SCHEPER (1989). Berlin, Heidelberg: Springer-Verlag Pp. 613. ISBN Nos 3-540-50568-7, 0-387-50568-7.

This book is a representation of the current extent of research and progress in the field of contact dermatitis and environmental dermatitis in Europe, and beyond. It is a collation of the proceedings of the inaugural meeting of the European Society of Contact Dermatitis (ESCD), held in Heidelberg in May, 1988. This meeting was held under the direction of the European Environmental and Contact Dermatitis Research Group (EECDRG), supported by the International Contact Dermatitis Research Group (ICDRG).

Contributions from all aspects of contact dermatitis investigation and research are represented in this volume, and the editors have categorized the papers into sections with common interest, e.g. immunological aspects, morphological aspects, current contact allergens, irritant contact dermatitis, contact urticaria, photosensitizers, diagnostic methods and preventive measures.

The contents of each paper are accurately detailed, with illustrations, both graphic and photographic, where relevant, and all papers were scrutinized by members of the European Group prior to oral delivery and also prior to publication in this volume. This therefore represents an update on current research rather than a basic guide to newcomers to the field of contact dermatitis, but it is a highly desirable volume for all departments of dermatology to keep abreast of the huge variety of aspects of research in this rapidly developing field.

A.FORSYTH

British Journal of Dermatology (1990) **122**, 721.

News and Notices

Fourth International Symposium on Pediatric Dermatology
Mazara del Vallo (Trapani), Italy, 25–28 September 1991

The Fourth International Symposium on Pediatric Dermatology, organized by the Gaspare Morello International Center for Study and Research and the Foundation for Research in Dermatology, will be held at Mazara del Vallo, Italy on 25–28 September 1991. For further information contact Dr Giuseppe Fabrizi, Scientific Program Director, Department of Dermatology, Catholic University, Largo Gemelli 8, 00168 Rome, Italy.

Mexican Society of Dermatopathology

The Sociedad Mexicana de Dermatopatologia A.C. has been organized and held its first meeting on 20 May 1989.

Melanoma Study Group
1990 Summer Meeting

A meeting will be held of the Melanoma Study Group on Friday 1 June 1990 at the Leicester Royal Infirmary. For further details write to Dr Robin Graham-Brown, c/o Postgraduate Centre, Leicester Royal Infirmary, Leicester.

British Journal of Dermatology (1990) **122**, 722.

Erratum

Graham-Brown RAC, Osborne JE, London SP *et al.* The initial effects on workload and outcome of a public education campaign on early diagnosis and treatment of malignant melanoma in Leicestershire. *Br J Dermatol* 1990; **122:** 53–59.

Table 1 of this article had a mistake as regards the number of melanomas seen in 1985. This should now read 35 and not 41.

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Antigens of the major histocompatibility complex in patients with chronic discoid lupus erythematosus

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SUMMARY

The frequencies of the major histocompatability complex class I, class II and class III antigens were determined in 130 patients (88 women and 42 men) with chronic discoid lupus erythematosus, and compared with those of 764 healthy controls. A significant increase in HLA-B7 (38·0% in the patients vs. 25·8% in the control group), HLA-B8 (29·5% vs. 17·4%), HLA-Cw7 (58·9% vs. 26·1%), HLA-DR2 (46·9% vs. 29·7%), HLA-DR3 (32·0% vs. 19·4%), HLA-DQw1 (76·6% vs. 60·5%), and a decrease in HLA-A2 (41·9% vs. 55·7%) was found. The calculated relative risk values for the respective antigens markedly increased when two or more antigens were present in one patient, with a maximum relative risk value of 7·4 for the combinations of HLA-Cw7, DR3, DQw1 and HLA-B7, Cw7 and DR3, which were found in 17·2% of the patients and in only 2·3% of the controls.

Twin and family studies of lupus erythematosus (LE) have shown that besides environmental factors which are necessary for full expression of the disease, genetic factors play a significant role.[1] Certain determinants encoded by the major histocompatibility complex (MHC), such as HLA-DR3 and probably the silent alleles of the complement component C4, are associated with systemic lupus erythematosus.[2,3]

Cutaneous LE is a disease with a broad clinical spectrum and several variants can be differentiated, such as discoid LE (CDLE), disseminated discoid LE, LE tumidus, LE profundus, bullous LE and subacute cutaneous LE (SCLE). CDLE is characterized by sharply defined persistent scaly plaques which heal with scarring and are preferentially localized on the face, the scalp and the neck. Association with systemic disease is uncommon[4] and it can be differentiated by histopathology from SLE as well as from the other variants of cutaneous LE.[5]

There have been few studies of the frequency of the major histocompatibility complex (MHC) determinants in CDLE.[6–8] We have analysed the class I (HLA-A, B, C), class II

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(HLA-DR, DQ) and class III (complement components C4A, C4B, Bf) determinants in 130 patients with confirmed CDLE, and compared the phenotypic frequencies with those of healthy controls.

METHODS

Patients

One hundred and thirty Caucasian patients (88 women and 42 men) with CDLE were studied. Only patients with typical discoid, hyperkeratotic and scarring lesions of CDLE confirmed by histology and immunofluorescence entered the study. Patients with lesions indicative for other variants of LE (SCLE or SLE) and those with hair loss, photosensitivity, arthritis, arthralgia, pleurisy or pericarditis were excluded. None of these patients had those criteria of the American Rheumatism Association for SLE.[9]

The following routine laboratory investigations were performed: erythrocyte sedimentation rate, white blood cell count and differential, urinalysis, creatinine, and serum transaminases. Additionally, the patients were tested for rheumatoid factor, syphilis, antinuclear antibodies, antibodies towards extractable nuclear antigens (RNP, SM), single-stranded and double-stranded DNA, immunoglobulin concentrations, complement component C3 and C4 by radial immunodiffusion and total haemolytic complement (CH50).

Controls

Seven hundred and sixty-four Caucasian individuals from the same area as our patients were typed for HLA-A, B, C, DR, DQ determinants and 113 were also typed for their complement allotypes Bf, C4A, and C4B according to established methods.[10–12]

Statistical evaluation

The phenotype frequencies of the HLA, Bf, C4A and C4B systems were compared using contingency tables (Fisher's exact test, where necessary) with corrections according to the number of alleles tested.[13] Relative risk values (RR) were calculated according to the method of Woolf.[14]

RESULTS

The frequencies of the HLA-A antigens showed a statistically significant decrease of HLA-A2 among the patients (41·9%) compared to controls (55·7%) ($\chi^2 = 8{\cdot}4$; $P_{\mathrm{corr}} = 0{\cdot}05$). The frequencies of the HLA-B locus products are shown in Table 1. The HLA-B7 and HLA-B8 antigen frequencies were elevated in the patient group: $\chi^2 = 12{\cdot}3$; $P_{\mathrm{corr}} = 0{\cdot}05$ for HLA-B7, and $\chi^2 = 10{\cdot}4$; $P_{\mathrm{corr}} = 0{\cdot}05$ for HLA-B8. No significant difference was observed with the other HLA-B antigens. The frequencies of HLA-C locus antigens (Table 2) revealed an increase in HLA-Cw7 in the patients (59·2%) compared to controls (24·6%) ($\chi^2 = 55{\cdot}9$; $P_{\mathrm{corr}} = 0{\cdot}001$).

With the HLA class II alloantigens (HLA-DR, DQ), significant differencies were found for HLA-DR2, HLA-DR3 and HLA-DQw1 (Table 3). The HLA-DR2 antigen was present in 46·9% of CDLE cases versus 29·7% of the controls ($\chi^2 = 14{\cdot}3$; $P_{\mathrm{corr}} = 0{\cdot}005$), HLA-DR3 was found in 32·0% versus 19·2% ($\chi^2 = 10{\cdot}5$; $P_{\mathrm{corr}} = 0{\cdot}05$), and HLA-DQw1 in 76·6% versus 60·5% of the controls ($\chi^2 = 12{\cdot}1$; $P_{\mathrm{corr}} = 0{\cdot}005$).

The frequencies of the allotypic variants of the complement components Bf, C4A and C4B showed no differences between patients and controls (data not shown).

The calculated relative risk (RR) values for these negatively or positively associated antigens,

TABLE 1. HLA-B antigen frequencies (%) in CDLE patients and in healthy controls

HLA	CDLE ($n=130$)	Controls ($n=764$)	χ^2	P_{corr}
B5	8·5	13·8		
B7	38·0	25·8	12·3	0·05
B8	29·5	17·4	10·4	0·05
B12	15·5	24·6		
B13	5·4	6·0		
B14	3·9	2·4		
B15	11·6	20·2		
B16	2·3	7·1		
B17	5·4	8·5		
B18	13·2	8·4		
B21	2·3	2·5		
B22	8·5	4·2		
B27	4·7	9·8		
B35	21·7	15·6		
B37	2·3	2·6		
B40	10·1	13·0		
B41	0·8	2·6		
B47	1·6	0·7		

HLA-A2, B7, B8, Cw7, DR2, DR3 and DQw1, are given in Table 4. The highest RR value for a single antigen was found for HLA-Cw7 with 4·7. However, the RR value rose to 7·4 for the combination of HLA-Cw7, HLA-DR3 and HLA-DQw1 in the same individual. This combination was found in 17·2% of the patients compared to 2·3% of the controls. Similar results were obtained for the combination HLA-B7, HLA-B8, and HLA-Cw7, found in 6·5% of the CDLE versus 2·3% of the controls. All RR values given in Table 4 are statistically significant.

The laboratory evaluations revealed no important abnormalities: 64% of the patients were ANA-positive, and anti-dsDNA and SM antibodies were negative in all the patients.

TABLE 2. HLA-C antigen frequencies (%) in CDLE patients and in healthy controls

HLA	CDLE ($n=130$)	Controls ($n=764$)	χ^2	P_{corr}
Cw1	4·7	8·5		
Cw2	6·2	9·3		
Cw3	21·7	29·6		
Cw4	17·8	19·0		
Cw5	5·4	9·7		
Cw6	14·7	16·0		
Cw7	58·9	26·1	55·9	0·001
Cw8	3·1	1·7		

TABLE 3. HLA class II antigen frequencies (%) in CDLE patients and in healthy controls

HLA	CDLE ($n = 128$)	Controls ($n = 764$)	χ^2	P_{corr}
DR1	14·1	18·7		
DR2	46·9	29·7	14·3	0·005
DR3	32·0	19·4	10·5	0·05
DR4	14·8	26·2		
DR5	28·9	26·4		
DRw6	25·0	20·4		
DR7	14·1	22·4		
DRw8	5·5	5·9		
DRw9	0·8	2·6		
DRw10	0·8	1·3		
DQw1	76·6	60·5	12·1	0·005
DQw2	45·3	39·4		
DQw3	40·6	56·0		

TABLE 4. Relative risk values (RR) for one and more HLA antigens expressed in CDLE patients vs. healthy controls

	RR	P_{corr}
One antigen	2·5	0·05
HLA-B7	1·8	0·05
HLA-B8	2·0	0·05
HLA-Cw7	4·2	0·001
HLA-DR2	2·1	0·005
HLA-DR3	2·0	0·05
HLA-DQw1	2·1	0·05
Two antigens	2·8	0·0001
HLA-B7, DR2	1·9	0·05
HLA-B8, DR3	2·7	0·0001
HLA-B7, B8	3·7	0·0001
HLA-B7, Cw7	3·9	0·0001
HLA-Cw7, DR2	3·9	0·0001
HLA-B8, Cw7	4·5	0·0001
Three antigens	3·1	0·0001
HLA-B8, Cw7, DR3	4·4	0·0001
HLA-B7, B8, Cw7	7·1	0·0001
HLA-B7, Cw7, DR3	7·4	0·0001
HLA-Cw7, DR3, DQw1	7·4	0·0001

DISCUSSION

The variants of LE have been shown to be heterogeneous, particularly in their immunogenetic background.[15-19] In the present study a large number of CDLE patients were analysed and this subtype of LE was shown to be positively associated with HLA-B7, B8, Cw7, DR2, DR3 and DQw1. The respective RR values for these antigens averages about 2·0 with a maximum value of 4·2 for HLA-Cw7. A combination of the above antigens in one individual raised the RR value to 7·4 for HLA-Cw7, DR3 and DQw1 and for HLA-B7, HLA-Cw7 and DR3. These specific HLA antigen combinations were found in 17·2% of the CDLE patients compared to 2·7% of controls.

To our knowledge, there have been few HLA studies in CDLE patients. In a recent study with 19 black and 22 white CDLE patients, an increase of HLA-DRw6 was reported in both races as compared with controls.[7] Other CDLE studies demonstrated an association with HLA-A28 in black patients and with HLA-B5 in white patients,[20] with HLA-B8 in patients greater than 40 years old,[6] or no significant association.[8] In a study of HLA antigens in 69 patients with CDLE,[6] an increased incidence of HLA-B7 and HLA-B8 related to the age at onset and sex of the patients was reported. Female patients, with onset 15–39 years, with either SLE or CDLE which had transformed to SLE, showed a significant increase in HLA-B8 compared with the females in the same age at onset group who had CDLE. We did not investigate this particular relationship, but the follow-up of our patients for 2–8 years has not revealed transformation into SLE and in most cases the ANA titres have remained constant over this time.

The results presented demonstrate the distinct immunogenetical background of CDLE compared to other types of LE. In most studies of SLE patients an increased frequency of HLA-DR3 has been found,[18,19,21-23] while in a few an increase in HLA-DR2 has been described.[1,2] In the case of drug-induced LE, e.g. hydralazine-induced LE, an association with the HLA-DR4 antigen was found.[24] Recent data on Venopyronum-induced LE showed an association with HLA-DR4 and the Gm 3.5.12 allotype, and a negative association with HLA-DR3.[25] In another recently defined subset of LE, subacute cutaneous LE, characterized by distinct non-scarring annular of papulosquamous lesions accompanied by few or no serious systemic features,[26] HLA-DR3 was found to be increased.[27] A partial or complete lack of the serum complement component C4 due to one or more null alleles at the C4A and/or CB4 loci has been reported to be associated with SLE, and is thought to increase susceptibility to SLE.[28] In our study of CDLE patients no increase of C4A of C4B null alleles was found.

The immunogenetic data in CDLE suggest a combined effect of distinct HLA class I and class II gene products on susceptibility to CDLE. However, the relatively low RR value for a single HLA antigen reflects the involvement of other polymorphic structures such as immunoglobulins and/or T-cell receptor molecules. Molecular genetical analysis of, for example, T-cell receptor polymorphism, may further elucidate the genetic background of susceptibility to develop CDLE.

ACKNOWLEDGMENT

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Nickel–keratinocyte interaction: a possible role in sensitization

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SUMMARY

Normal human keratinocytes and the keratinocyte-derived cell lines NCTC 2544 and A 431, were exposed for different periods (1–5 days) to various concentrations (0·023–46·6 μg/ml) of nickel (Ni^{2+}). A dose- and time-dependent inhibition of cell growth and viability was observed. Cultures exposed to 2·3 μg Ni^{2+}/ml showed approximately 50% cell survival at 5 days. An increase in release of interleukin 1 by keratinocytes was detected following culture for 24 h with a Ni^{2+} concentration of 2·3–11·5 μg/ml. Short periods of incubation (30 min) with these concentrations induced an activation of lipoxygenase in leucocytes from healthy subjects, without modifying cell viability. The results suggest that the percutaneous penetration of small amounts of Ni^{2+} can result in damage to keratinocytes and can initiate sensitization.

Nickel is one of the causes of contact dermatitis, and in recent years there has been an increased incidence of patch-test positive patients.[1-3] The main source of the allergen is jewellery, particularly ear-rings.[1-4] Recent reports have indicated that nickel-containing ear-rings release nickel ions with different leaching solvents, although the amounts vary greatly due to their different compositions.[2-4] The minimal concentration of nickel released by ear-rings that can produce an allergic reaction in sensitized subjects, has been calculated at about 0·1 μg/cm^2 per day.[2]

The crucial role of the epidermal antigen-presenting cells in inducing nickel hypersensitivity has been shown, and the amount of nickel retained in the tissues is considered fundamental in the maintenance of the cutaneous manifestations.[5-7] Nickel ions bind with human serum albumin and some amino acids, such as histidine, and their uptake by different cells has been reported both *in vitro* and *in vivo*.[8-11] When nickel is applied to the skin some remains in the horny layer, but it also penetrates deeper into the sweat ducts.[12] The application of 2·5% or 1% nickel sulphate by patch tests on normal subjects in whom the uppermost layers of epidermis have been damaged results in acute inflammatory reaction.[12]

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It has been shown previously that the amounts of Ni^{2+} released by nine samples of different clips and clasps, maintained in an artifical sweat at pH 6·5, varied between 49 and 101 μg/12 h.[3] Even if there is less absorption, it is likely that the continuous wearing of earrings releases a high amount of Ni^{2+}, which persists for a long time and can induce sensitization.

The aim of the present study was to evaluate the effect of several Ni^{2+} concentrations on cell viability and the production of interleukin 1 (IL-1), using human keratinocytes, as well as their activation of lipoxygenase of leucocytes from normal volunteers.

METHODS

Cell cultures

Keratinocyte cultures were derived from neonatal foreskin and plated on feeder layers of mitomycin C-treated 3T3 fibroblasts.[14] Cells were cultured in 24-well plates (Flow, Irvine, Scotland, U.K.) and incubated using Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland, U.K.) with 10% fetal bovine serum (FBS), 0·4 μg/ml hydrocortisone, 1 nM cholera toxin, insulin 5 μg/ml, transferrin 5 mg/ml, triiodothyronine 2×10^{-9} M, glutamine and antibiotics; the medium contained 0·1 mM calcium. Cells growing in such medium do not cornify but continue to proliferate.[15] The keratinocytes were used at the second or third passage which gave more consistency as to the number of cells in each well ($4·5 \times 10^5$). In preliminary experiments, the human keratinocyte-derived cell line NCTC 2544 (Flow) and human squamous carcinoma-derived cell line A 431, seeded at $2–3 \times 10^5$ cells/well, were also used.

Experimental conditions

The concentrations of nickel sulphate ($NiSO_4 \times 6H_2O$, Merck AG, Darmstadt, F.R.G.) ranging from 0·1 to 200 μg/ml, corresponding to 0·023–46·6 μg Ni^{2+}/ml were added to the cultures. After 1–5 days cells were pulsed with 0·5 μCi [^{3}H]-thymidine (6·7 mCi/mM, Dupont, Boston, MA, U.S.A.) for 24 h, precipitated in 10% trichloroacetic acid, resuspended in NaOH 1N and the radioactivity evaluated by liquid scintillation counting. All the tests were performed in triplicate and the results are reported as mean ± SD of at least four experiments. The values are expressed as Ni^{2+} concentrations.

IL-1 production

Human foreskin keratinocytes were cultured in serum-free medium ($4–5 \times 10^5$ cells/well) in the presence of 0·23–11·5 μg Ni^{2+}/ml. After 24 h the supernatants were collected, filtered, dialysed and the ability to co-stimulate the mitogenic activity of suboptimal concentrations of Concanavalin-A (Con-A, 0·5 μg/ml) (thymocytes co-stimulatory test) on thymocytes from C57/b mice, was evaluated.[16]

Lipoxygenase activity

Human cultured keratinocytes contain a lipoxygenase activity similar to that of leucocytes.[17] The effect of nickel in stimulating the enzyme was evaluated on the more easily available leucocytes. Polymorphonuclear cells (PMN) (2×10^6/ml) were obtained from five normal volunteers and pre-incubated with $NiSO_4$ (2·3–23·3 Ni^{2+} μg/ml) in phosphate buffered-saline (PBS, Gibco) at pH 7·4 for 30 min. Arachidonic acid (50 μg/ml) (C20:4) or linoleic acid (C18:2) in ethanol were then added. The cells were incubated at 37 °C for 5–10 min, centrifuged and the supernatants extracted three times with chloroform:methanol (2:1), with 50 μg C16:0 as internal standard. The solvent was evaporated under nitrogen and the fatty acids methylated with boron trifluoride (10% in MeOH) (Merck). Fatty acid methyl esters were quantified by gas chromatography on FFA-P capillary column (50 mm × 0·32 mm ID, Hewlett–Packard). The results are reported as the percentage decrease of the fatty acids and represent the mean of three experiments in triplicate.

RESULTS

Viability of keratinocytes

Nickel ions at concentrations between 0·23 and 46·6 μg/ml showed a cytotoxic effect on human foreskin keratinocytes in culture, as well as on the cell lines used. The effect was time- and dose-dependent; a progressive reduction in cell growth and viability was observed as the period of exposure was increased (Figs 1 and 2) even at the concentrations that had no effect at 24 h. This was more evident in cell lines increasingly susceptible to the ions.

Production of IL-1

The effect of the keratinocyte supernatants, treated for 24 h with different concentrations of Ni^{2+}, on the thymocyte co-stimulatory test is shown in Figure 3. A co-stimulatory effect in the presence of suboptimal concentrations of Con-A was found which correlated with the concentration of Ni^{2+}. The dialysis of the supernatants performed before the tests, excluded the possibility that the presence of Ni^{2+} induced a stimulation of thymocyte mitosis. Following 24 h of culture, the viability of human keratinocytes was not significantly affected by doses of nickel up to 4·6 μg Ni^{2+}/ml, whereas at the highest concentration employed (11·5 μg Ni^{2+}/ml) a slight toxicity (20 ± 5% mortality) was observed (Fig. 1). These results suggest a direct effect of the ions on IL-1 production, rather than a non-specific release due to significant cell damage and lysis.

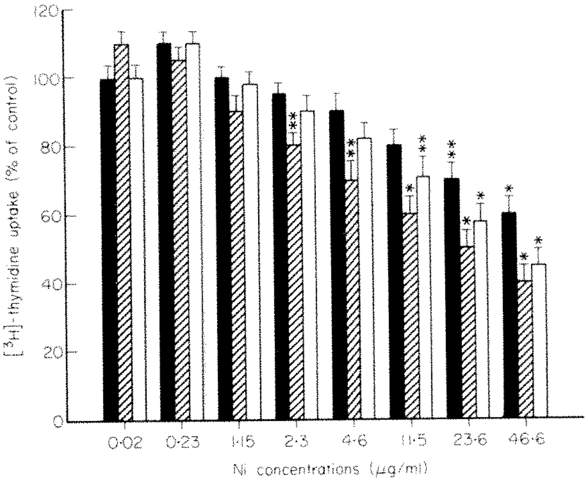

FIGURE 1. Dose-dependent effect of different concentrations of nickel ions on viability (at 24 h) of human foreskin keratinocytes (HK) (■), NCTC 2544 (▨) and A431 cell lines (□). Each result represents the mean ± SD of four experiments in triplicate.** $P < 0{\cdot}05$; * $P < 0{\cdot}01$.

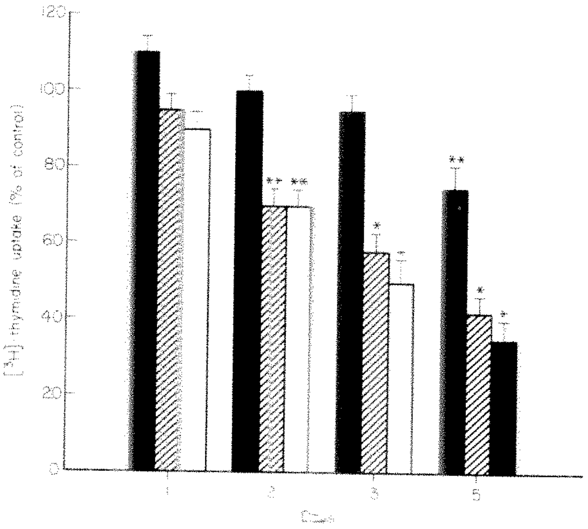

FIGURE 2. Time-dependent effect of nickel ions on viability of human keratinocytes. Each result represents the mean ± SD of four experiments in triplicate. ** $P < 0·05$; * $P < 0·01$. Nickel concentrations (μg/ml): ■, 0·23; ▨, 2·3; □, 4·6.

Activation of lipoxygenase

Polymorphonuclear cells incubated with Ni^{2+} for 30 min were able to oxidize arachidonic acid or linoleic acid, the natural substrates of lipoxygenase, at a rate significantly higher than controls. The rate of decomposition was dependent on the concentrations of nickel added. The results are shown in Figure 4 as the percentage decrease of the concentration of fatty acids. The

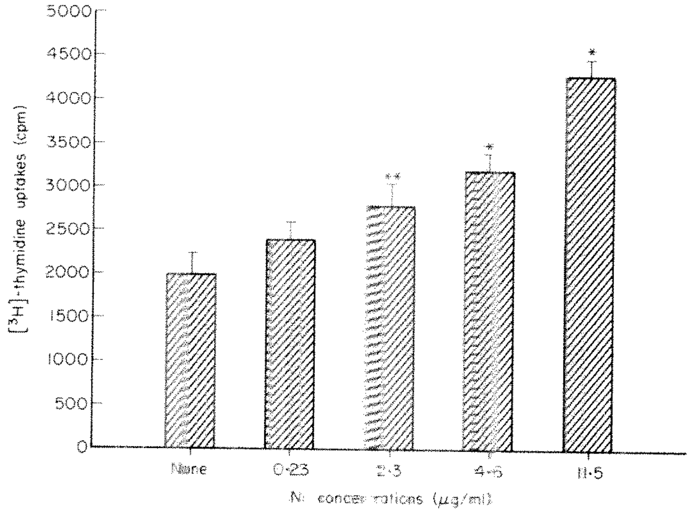

FIGURE 3. Effects of supernatants of human keratinocytes cultivated with different concentrations of nickel ions for 24 h, in the thymocyte co-stimulatory tests. Results represent the mean ± SD of three experiments in triplicate. ** $P < 0·05$; * $P < 0·01$.

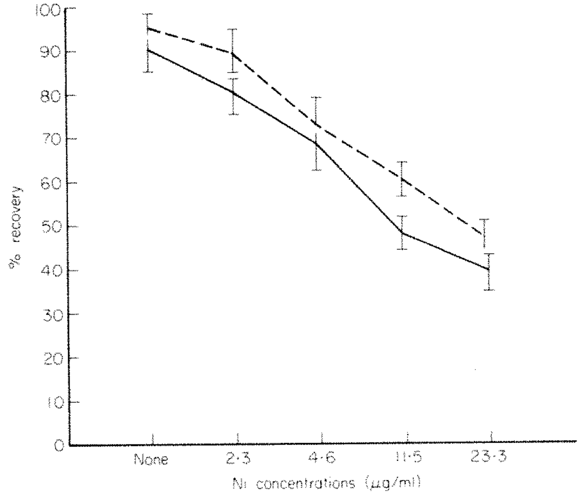

FIGURE 4. Rate of decomposition of arachidonic acid (C20:4) (——) and linoleic acid (C18:2) (- - -) incubated with 10^6 PMN in the absence or presence of different concentrations of nickel ions. Each result represents the mean ± SD of three experiments performed in triplicate.

effect was inhibited by nordehydroguaieretic acid (10^{-5} M), a specific inhibitor of lipoxygenase,[17] indicating that lipoxygenase was activated. To exclude the non-specific release of the enzyme due to cell death, the cells were washed and cultured for up to 24 h in RPMI 1640 supplemented with 10% fetal calf serum and their viability evaluated by the trypan blue exclusion test. There was no significant difference from the controls with concentrations up to 11·5 μg Ni^{2+}/ml.

DISCUSSION

Exposure to nickel is considered a health hazard,[10] the ions can bind to various cell types and can cause cancer.[9-12] The skin manifestations induced by nickel are mainly eczematous reactions and the incidence of sensitization is increasing. In 1988 64% of our subjects who were positive to at least one allergen of the ICDRG (International Contact Dermatitis Research Group) standard series, were found to have a positive patch-test response to 5% nickel sulphate and about 98% of patients with earlobe dermatitis have the same positivity.[3]

The role of a direct effect of the applied hapten on keratinocyte viability and function in inducing sensitization has not been completely evaluated. However, the period of exposure and the amount of hapten applied, together with the function of the epidermal dendritic cells are considered of fundamental importance.[2,5,6] Probably both the production of an inflammatory reaction and the activation of different epidermal constituents are important steps.

Our results show that concentration of Ni^{2+} between 0·23 and 46·6 μg, lower than those released by samples of metal clips and clasps, can interfere with keratinocyte growth and viability in a dose- and time-dependent manner. After 24 h of culture, the lowest concentrations used can increase IL-1 production by keratinocytes. IL-1 induces lymphocyte activation and the subsequent release of IL-2 and γ-interferon,[16] all events which can initiate a cascade resulting in an inflammatory process. The ions stimulate the lipoxygenase pathway, with generation of hydroperoxides, allowing the chemotaxis of leucocytes, and in turn, an increase of inflammatory mediators.

The mechanisms of the activation of lipoxygenase and the stimulation of IL-1 production by nickel is not clear. It could be due to membrane damage, or to a direct effect of the ions on the enzymes of the cell membrane. An increase in intracellular calcium is a common mechanism of cell injury and death, and calcium can activate phospholipases.[18] The stimulation of the release of cytokines by epidermal cells has been correlated with the presence of hydroperoxides and with cell changes.[16] It was suggested that barrier damage in the stratum corneum, or inflammatory products, can induce morphological signs of Langerhans cell activation.[19]

In a previous paper[3] the amounts of Ni^{2+} released by metal clips and clasps were evaluated, and the results showed that these were significantly higher than the normal daily dietary intake.[10] The cutaneous intake from clips and clasps is not at physiological levels and cutaneous adaptivity similar to that described for intestinal absorption is not likely.[3,20]

Nickel ions can bind to human epidermis and this layer can act as a local reservoir.[21,22] The absorption by the epidermis increases with repeated contact with the metal because of the presence of other factors.[1-3] Fullerton and Hoelgaard[22] found that in the range of 0·5–200 μg/ml about 90% of the available nickel bound to the epidermis, and they suggested that when applied to the skin, there is penetration of the ion across the stratum corneum. The concentration of nickel *in situ* gradually increases and a greater proportion of nickel is free to permeate through the epidermis. In addition, the mean retention time of nickel in tissues is hundreds of days,[13] so that a significant amount of the metal remains on the skin, following the continuous use of earrings. The concentration of the metal used in *in vitro* experiments is similar to that found *in vivo*.

These results suggest that the direct toxic effect on the epidermis, the generation of inflammatory mediators and the stimulation of IL-1 production could be the primary events in causing nickel sensitization.

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Adult mastocytosis: an immunophenotypic and flow-cytometric investigation

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SUMMARY

Skin mast cells of five patients with mastocytosis/urticaria pigmentosa and from four normal controls were immunophenotyped with a panel of 17 monoclonal antibodies; peripheral blood mononuclear cells from the patients were similarly labelled and analysed by flow cytometry. Cutaneous mast cells expressed leucocyte common antigen and markers of macrophage/monocyte lineage suggesting a common progenitor in the ontogeny of the two cell types. The mast cells from the patients differed from the controls, expressing HLA-DR in four cases, and this may be a feature of proliferating cells. The patients' peripheral blood T lymphocytes showed a marked increase in the activation markers HLA-DR and CD25 suggesting a contributory role, possibly via cytokines, in driving mast-cell proliferation.

The immunophenotypic expression of markers associated with mast cells have been subject only to limited investigation.[1,2] The use of monoclonal antibodies (MAb) to identify the immunophenotype of proliferating mast cells in urticaria pigmentosa may help to elucidate not only the ontogeny and differentiation of cutaneous mast cells, but also the degree of atypia of such proliferative disorders. As *in vitro* studies suggest that mast-cell proliferation is in part dependent upon factors derived from inducer T lymphocytes,[3,4] aberrant T-cell regulation of mast-cell production might play a significant role in the pathogenesis of urticaria pigmentosa. A study of five adult patients with extensive urticaria pigmentosa was undertaken to establish the immunophenotype of lesional and normal cutaneous mast cells. Immunophenotyping of the patient's peripheral blood cells was also carried out to document the nature of the T cells.

METHODS

Patients

Five patients with extensive and histologically confirmed urticaria pigmentosa of adult onset were studied. Their clinical details are summarized in Table 1. All had extensive skin lesions and experienced flushing as well as pruritus, with Patients 1 and 2 having evidence of systemic

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TABLE 1. Clinical features of patients studied

Patient	Age	Sex	Duration (years)	Type	Other organs involved	Associations
1	77	M	2	Nodular/papular	Eosinophils $0{\cdot}22 \times 10^9$, bone marrow and spleen	Flushing, pruritus, rapid progression
2	50	F	20	TEMP	Liver, small intestine, bone, bone marrow	Eosinophilia $2{\cdot}5 \times 10^9$/l, basophilia $0{\cdot}55 \times 10^9$/l, flushing, pruritus, diarrhoea
3	53	M	1·5	TEMP	—	Flushing, pruritus
4	50	F	7	UP	—	Flushing, pruritus
5	40	F	11	UP	—	Hymenoptera anaphylaxis, flushing, pruritus

TEMP, telangiectasia eruptiva macularis perstans. UP, urticaria pigmentosa.

involvement. Patient 1 had nodular and papular skin lesions of rapid onset (at least 1000 lesions developing over a 2-year period) with the mast cells in his bone marrow and skin lesions being larger than normal and atypical. He also developed moderate splenomegaly. Patient 2 had evidence of mast-cell infiltration of the liver, small bowel, bones and bone marrow. Patient 5 experienced recurrent anaphylaxis following bee stings as described by Muller.[5]

Treatment of the patients depended on symptoms and included H_1 and H_2 antagonists, non-steroidal anti-inflammatory drugs and sodium cromoglycate. As mast-cell proliferation may result from lymphokines, prednisolone was tried in Patient 1 (60 mg reducing to 15 mg daily over 6 weeks and stopping after 12 weeks) and in Patient 2 (40 mg for 2 weeks tailing to 0 over 6 weeks). Cyclosporin was also tried in Patient 1 at a dose of 5 mg/kg for 1 month, achieving trough levels of 191 μg/l. Equivalent lesional biopsies were taken before and after each of these interventions and numbers of mast cells per high powered field were counted.

Immunophenotyping of mast cells

Skin biopsies were taken from lesions from all five patients and from the normal skin of four healthy subjects. Specimens were placed in OCT medium (Tissue-Tek) and snap frozen in liquid nitrogen. Frozen sections were prepared at 6–8 μm thickness, air-dried at room temperature for 1 h and then fixed in cold acetone for 5 min after which they were stained separately by a standard indirect immunoperoxidase method (IPX) and by an unlabelled antibody bridge APAAP immunoalkaline phosphatase technique as previously described[6,7] using the panel of MAb shown in Table 2. (APAAP complexes were obtained from Dakopatts, Copenhagen.) The substrates used were respectively 3,3 diaminobenzidine, tetrahydrochloride and naphthol AS-MX/fast red TR.

After processing, all specimens were examined by light microscopy and the presence of brown (IPX) and fast red APAAP reaction products classified on a three-point scale: minus, definitely absent; ±, doubtful presence; +, definitely present. A classification of ± was only used if the presence of antigen was still doubtful after application of techniques for enhancement of APAAP labelling.[6] Serial sections from all specimens were stained with haematoxylin and eosin and with toluidine blue to allow the localization of mast cells.

TABLE 2. Monoclonal antibodies used for immunocytochemical staining of skin biopsies

Antibody	Source	Clone	Specificity
CD1	Dako	NA 1/34	Cortical thymocytes, Langerhans cells
CD3	Oxoid	UCHT-1	T-cells
CD4	Dako	T3-10	Helper/inducer T-cells/ monocytes and macrophages
CD7	Dako	DK24	T-cells
CD8	Sapu	RFT-8	Cytotoxic/suppressor T-cell
CD14	Serotec	BRC-M1/MA5	Macrophages/monocytes
CD15	Dako	C3D-1	Neutrophils, myelocytes
CD19	Dako	HD37	B-cells
CD22	Dako	4KB128	B-cells
CD23	Dako	MHM6	Activated B-cells/low affinity IgE Fc receptor
CD25	Dako	ACT-1	Activated T and B cells, NK cells, macrophages
CD45 antigen	Dako	ZB11 and PD7/26	Pan leucocyte common
HLA-DR	Dako	TAL.1B5	B-cells, activated T-cells antigen presenting cells
Dako-macrophage	Dako	EBM11	Macrophages
Myeloid/histiocyte	Dako	MAC387	Macrophages, squamous epithelium, neutrophils
Mast cell	Dr Rimmer[2]	MCG35	Mast cells—cytoplasmic antigens
IgE	Oxoid	1109.1	

Immunophenotyping of blood mononuclear cells

When the biopsies were obtained (around 09.00) 20 ml of peripheral venous blood were taken into 6 ml ACD anticoagulant, and in the two patients with extensive disease (Patients 1 and 2) bone marrow samples were also aspirated.

The immunophenotype of Ficoll-isopaque (S.G. 1.077) separated blood mononuclear cells was characterized by an indirect immunofluorescence staining method and the stained cells quantified by the EPICS 'C' flow microcytofluorograph (Coulter Electronics, Luton U.K). The primary layer mouse MAb used were of the following specification: CD1–5, CD8, CD14, CD19, CD23, CD25 (TAC-interleukin-2 receptor), HLA-DR, IgM, κ and λ light chain. A second layer goat anti-mouse immunoglobulin–fluorescein-conjugated antibody (Becton Dickinson) was used and the cytometric analysis of lymphocytes was performed as previously described,[8] using the bitmapping facility of the flow cytometer to exclude monocytes and granulocytes by the physical parameters of forward and 90° light scatter.

T- and B-lymphocyte enumeration together with T-cell subsets and activation marker (CD25 and HLA-DR) analyses were used to derive the percentage and absolute number of cells positive for the markers. The results were compared to the normal population of ranges as previously described.[9]

RESULTS

In the lesional skin biopsies, localization of antigen in or on mast cells was indicated by a brown IPX or vivid red (APAAP) reaction product which contrasted strongly with the haematoxylin

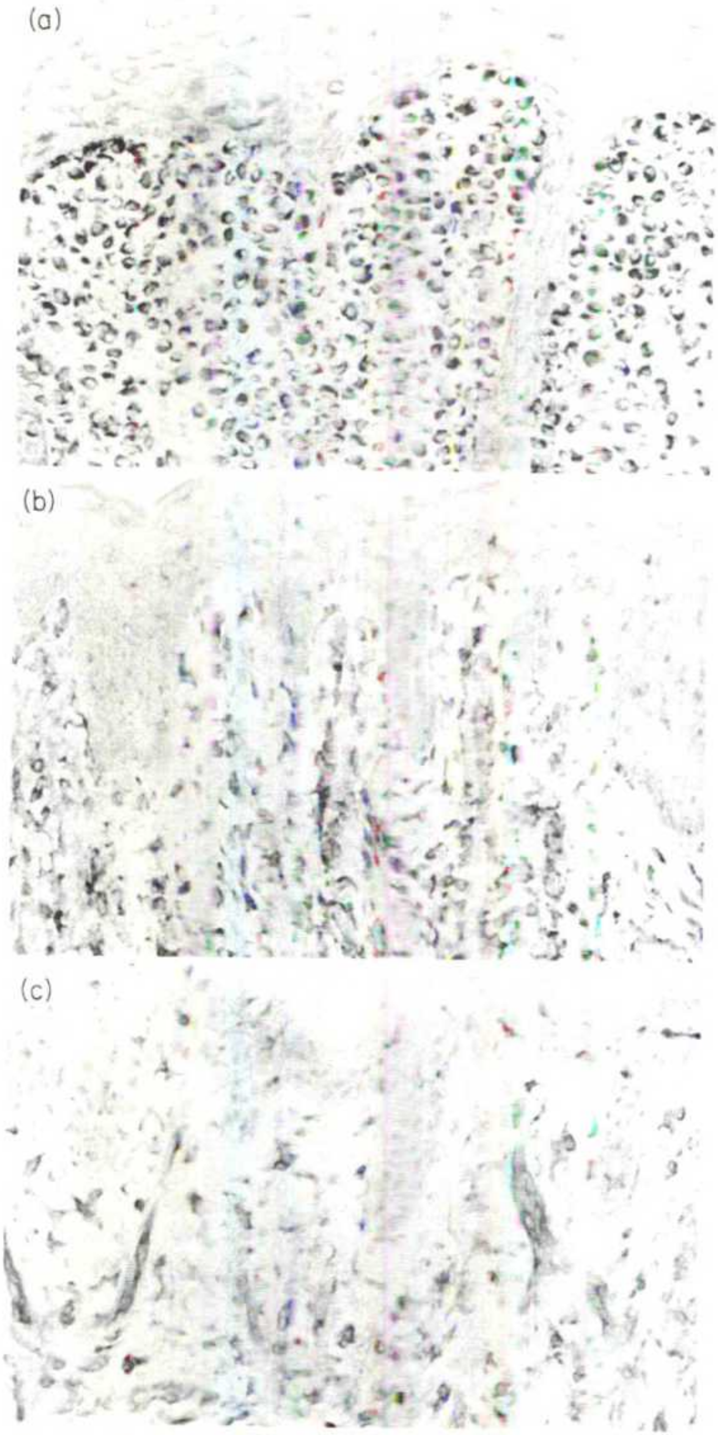

FIGURE 1. Lesional mast cells with immunoperoxidase substrate labelling. (a) Mast cell granules (Rimmer MCG 35); (b) Dako macrophage monoclonal; (c) HLA-DR (× 64).

TABLE 3. Phenotype of mast cells in lesional ($n = 5$) and normal skin biopsies ($n = 4$)

	Case					Normal
Antibody	1	2	3	4	5	(×4)
CD1	−	−	−	−	−	−
CD3	−	−	−	−	−	−
CD4	+	±	±	±	−	−
CD7	−	−	−	−	−	−
CD8	−	−	−	−	−	−
CD14	+	−	−	−	−	−
CD15	−	−	−	−	−	−
CD19	−	−	−	−	−	−
CD22	−	−	−	−	−	−
CD23	+	±	±	±	±	±
CD25	−	−	−	−	−	−
CD45	+	+	+	+	+	+
HLA-DR	−	+	+	+	+	−
Dako-macrophage	+	+	+	+	+	+
Myeloid/histiocyte	+	−	−	−	−	−
Mast cell (MCG35)	+	+	+	+	+	+
IgE	−	−	−	−	−	−

counterstain (Fig. 1). The results of staining with the immunophenotyping panel (Table 2) in the five patients and four control subjects are summarized in Table 3. Identical staining patterns using this panel were obtained with both immunocytochemical techniques in all nine biopsies.

Mast cells in the normal skin specimens stained consistently and strongly for leucocyte common antigen (CD45) and with a MAb known to bind specifically with granule components of human mast cells.[2] In addition strong positive staining was seen with a MAb directed against an unidentified antigen hitherto found only in cells of the monocyte/macrophage series (Dako-macrophage). Doubtful positive staining was also seen with an antibody against the CD23 antigen, a low affinity receptor for the Fc portion of IgE normally found on B cells in follicular mantle zones, on a subpopulation of dendritic reticulum cells and on peripheral blood B cells when stimulated *in vitro*.

In lesional skin from all five patients strong positive staining of mast cells was consistently found with antibodies against leucocyte common antigen (CD45), mast cell granules (Fig. 1a) and the unidentified antigen recognized by the Dako-macrophage MAb (Fig. 1b). The CD4 and CD23 antigens were doubtfully expressed by the mast cells of Cases 2–5 even after enhancement techniques. These mast cells also strongly expressed HLA-DR (Fig. 1c) in contrast to the normal cells. Patient 1, with more rapidly progressive disease, expressed an additional macrophage marker (the BRC monoclonal) and was positive for CD4 and CD23 but lacked HLA-DR expression.

In the peripheral blood, a consistent finding in all five patients was a relative and absolute increase in cells expressing the 'activation markers' CD25 and HLA-DR (Table 4). T lymphocytes were responsible for the increase in activation markers, as identified by bitmapping and the use of lymphocyte-specific antibodies. The percentage of cells positive for both

TABLE 4. Percentage of peripheral T-lymphocytes expressing activation markers CD25 and HLA-DR

Patient	1	2	3	4	5
CD25 ($n = 0$–3%)	19	11	11	15	46
HLA-DR ($n = 0\%$)	13	5	29	5	5

n, normal range.

activation markers was well in excess of total B cells (CD19 and/or IgM positive), which are known to constitutively express HLA-DR. Immunophenotypic findings for total T and B cells and subset ratios were within the normal range except in patient 2, who showed an increased CD4:CD8 ratio due to a relative and absolute increase in $CD4^+$ cells (67% CD4, 11% CD8). Mast cells were not identified in peripheral blood but if present their granularity causes light scatter similar to granulocytes and this would exclude them from the analysed lymphocyte bitmap. Immunophenotyping of the bone-marrow aspirates of Patients 1 and 2 revealed no significant abnormality apart from an increase in mast-cell numbers.

Immunosuppressive therapy with cyclosporin (Patient 1) and prednisolone (Patients 1 and 2) brought about no clinically detectable improvement except for relief of wealing with doses above 30 mg of prednisolone. Patient 1 showed no histometric improvement, but Patient 2 showed a marked fall (50%) in mast-cell numbers after 4 weeks of treatment, and her peripheral eosinophilia also fell from $2{\cdot}48 \times 10^9$/l to $0{\cdot}74 \times 10^9$/l.

DISCUSSION

Although the ontogeny of the mast cell is still uncertain, these results favour their origin from the pluripotential haematopoietic stem cell.[1,10,11] This is further supported by our finding of expression of leucocyte common antigen (CD45), as previously documented by Rimmer and Horton,[1] and the expression of Dako-macrophage positivity in lesional mast cells and normal skin mast cells suggests a common progenitor for both mast and macrophage/monocyte cell lines. Valent *et al.*[11] have recently published results of immunophenotyping of normal human mast cells from diverse anatomical sites including skin. Their findings were similar to those in our normal controls, and also demonstrated expression of markers of late stage macrophage/monocyte differentiation using different antibodies MAX 1,3,11 and 24. Identical results were found in lung, intestinal and peritoneal mast cells. Additional similarities between mast cells and macrophages include their presence predominantly in tissues and production of chymotrypsin and trypsin;[12] clinically there are also analogies between histiocytosis X and the mastocytoses[13] and, rarely, mast cells and histiocytes proliferate together in urticating histiocytosis.[14]

Immunophenotypically cells from our patients did not differ from normal mast cells apart from the expression of the HLA-DR in four of the five cases, which may reflect cell activation by lymphokines. The ability of mast cells to express HLA-DR has not to our knowledge been described and although seen in diverse cell types, is a further similarity with macrophages which constitutively express HLA-DR. The lack of HLA-DR expression in Patient 1 might be a reflection of disease heterogeneity.

The increase in activation markers on T cells may be important in the aetiology of mastocytosis by providing an increase in lymphokine drive to mast-cell progenitors; IL-3 (a mast-cell growth factor) and possibly other distinct lymphokines are important determinants of growth and differentiation of mast cells.[1,2,10,11,15] Recent research[16] has indicated that activated mast cells themselves produce IL-3, which induces mast-cell proliferation, and IL-4, which stimulates B cells which could then produce cytokines, resulting in an autocrine reciprocal drive of lymphocyte and mast-cell activation and proliferation. If this were the case then immunosuppression, particularly by cyclosporin A, a specific inhibitor of T-cell lymphokine production, may provide beneficial effects in mastocytosis by removing this drive. In the past, systemic steroids have produced variable results[17] but more recently potent topical steroids with occlusion have proved effective in reducing mast-cell numbers in the skin.[18] Although there was apparent diminution in lesional mast cells in Patient 2 following therapy with prednisolone, clinical improvement was unimpressive and sampling of different lesions could account for the reduction. Cyclosporin in high doses produced depletion of mucosal mast cells in a rat model,[19] but failed to influence lesions in Patient 1 after 4 months of treatment. These disappointing results may arise from too short a course given too late in the evolution of the disease. Alternatively the activated state of lymphocytes in our patients might be a secondary event with the contribution of cytokines from the large pool of mast cells in mastocytosis including IL-3 and IL-4.[16]

Mast-cell leukaemia and reticuloendothelial neoplasms, particularly leukaemia, occur more frequently in mastocytosis.[17,20] Proliferation of mastocytosis cells may be partly cytokine driven, but over time proliferating mast cells may become autonomous in a manner analogous to HTLV1-induced T-cell lymphoma, where IL-2 driven proliferation occurs for months or years prior to a clonal autonomous neoplasm.[21] It would be of interest to compare our results with the same markers in patients with malignant mastocytosis to assess whether markers will help to distinguish benign from malignant disease, although genotypic analysis for clonality should prove more useful.

The presence of a marked eosinophilia in Patient 2 is of interest and is a recognized feature in 12% of patients.[18] This could arise from an increase in T-cell IL-3 or IL-5 production[22] and respond to prednisolone. In chronic myeloid leukaemia, eosinophilia and basophilia are associated with a poor prognosis, and in systemic mastocytosis one-third of those with eosinophilia have, in one series, had a fatal outcome.[17] Perhaps such patients identified as being more at risk of neoplasia would benefit from long-term suppression of cytokine production by immunosuppressants.

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Neuropeptides in skin from patients with atopic dermatitis: an immunohistochemical study

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SUMMARY

The distribution and localization of several neuropeptides were investigated in the lichenified lesions of 11 patients with atopic dermatitis using indirect immunofluorescence. Substance P-positive nerve fibres were observed in most of the cases of atopic dermatitis, but not in normal controls. Somatostatin immunoreactive nerves were not found in the skin of atopic dermatitis, whereas a normal pattern of immunoreactivity could be detected in most of the healthy subjects. Neuropeptide Y-positive dendritic epidermal cells were observed in lesional skin from patients with atopic dermatitis, but not in controls. Calcitonin gene-related peptide and vasoactive intestinal polypeptide immunoreactivity in patients with atopic dermatitis did not differ from that in healthy subjects. With galanin antiserum a diffuse intracellular staining was observed in the epidermis of both atopic patients and controls, while no positive staining was found with either neurotensin or neurokinin A antibodies in either group. These findings suggest a possible involvement of some neuropeptides in the pathomechanisms of atopic dermatitis.

Atopic eczema is an inflammatory skin disease and exacerbations of the disease can be provoked by either immunological or non-immunological factors. The latter includes emotional stress, scratching and sweating, which tend to induce chronic inflammation. Neuropeptides (NP) which are known to play a crucial role in neurogenic inflammation, could be involved in these mechanisms. In particular, substance P (SP), neurokinin A (NKA) and calcitonin gene-related peptide (CGRP) have been shown to be the main mediators of flare and weal reactions induced through axon reflexes.[1-4] In addition, vasoactive intestinal polypeptide (VIP) regulates sweat secretion,[5] while SP, NKA, CGRP, VIP and Neuropeptide Y (NPY) are implicated in the regulation of skin blood flow.[4,6,7]

Recent studies have indicated an important role of NP in the pathogenesis of asthma.[8-10] A reduction in both flare and weal responses to intradermal injections of SP, NKA, NT and a reduced flare to CGRP injection have recently been observed in atopic dermatitis (AD).[11]

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Several NP have been demonstrated in nervous structures as well as cellular elements of human skin by immunohistochemistry and radioimmunoassay.[5,12-14] In the present study we investigated the distribution and localization of a number of NP in the skin of patients with AD.

METHODS

Patients

Eleven patients with AD, seven male and four female, age range 10–65 years, were studied. Skin biopsies were taken from lichenified areas on the flexures of the elbows. Control biopsies were taken from the flexures of the elbows from six healthy adult volunteers and from lesions of two patients with inflammatory dermatoses (generalized contact dermatitis and prurigo), neither of whom had any personal or family history of atopy.

Antisera

Polyclonal rabbit antisera directed against NPY, CGRP, VIP, NKA, neurotensin (NT), somatostatin (SOM) and galanin (GAL) and a rat monoclonal anti-SP antibody were used. Details of the antibodies are given in Table 1. As cross-reactivity with unknown peptides or other proteins present in tissue section cannot be excluded, the term 'NP-like immunoreactivity' will be used.

Immunohistochemistry

Punch biopsies (4 mm) were obtained under local anaesthesia. Tissue samples were immersed for 2 h in an ice-cold paraformaldehyde/picric acid solution and then rinsed for 24 h at 4°C in 0·1 M Sörensen's buffer containing 5% sucrose. Frozen sections (14 μm) were incubated overnight at 4°C in a humid atmosphere with the antisera (Table 1), and rinsed three times with phosphate buffered saline (PBS) for 10 min. They were then incubated for 30 min at 37°C with biotin-

TABLE 1. Details of peptide antibodies used

Peptide	Code	Working dilution	Source
Calcitonin gene-related peptide	2105-010	1:640	Byk-Gulden, MILAB, Malmö, Sweden
Neuropeptide Y	2117-010	1:100	Byk-Gulden, MILAB, Malmö, Sweden
Vasoactive intestinal peptide	RAS 7161-N	1:100	Peninsula Laboratories Europe Ltd, England
Substance P	MAS 035 (monoclonal)	1:100	SERALAB Ltd, Crawley Down, England
Somatostatin	6[illegible]019	1:80	Peninsula Laboratories Europe Ltd, England
Neurotensin	6[illegible]004	1:100	Peninsula Laboratories Europe Ltd, England
Neurokinin A	RAS 7359-N	1:100	Peninsula Laboratories Europe Ltd, England
Galanin	2109-010	1:160	Byk-Gulden, MILAB, Malmö, Sweden

labelled anti-rabbit serum (Vector Laboratories Inc., Burlingame, CA, U.S.A.) diluted 1:30, rinsed as before and finally incubated for 30 min at room temperature with fluorescein-isothiocyanate (FITC)-labelled streptavidin (Amersham International, England) diluted 1:50. A FITC-labelled anti-rat serum (Sera-Lab Ltd, England) diluted 1:5, was used for 30 min at 37°C as a secondary antibody for SP. After a further washing in PBS, slides were mounted in buffered glycerol. Controls were performed by incubating the sections with only the secondary antiserum, or by replacing the primary antiserum with a non-immunized rabbit or rat serum. No specific staining was observed in the controls. All solutions contained 0·3% Triton X-100 and 3% goat serum. The sections were studied and photographed under a Leitz fluorescence microscope.

RESULTS

SP-like immunoreactivity was strongly expressed in the skin of nine out of 11 AD patients and in the case of prurigo. Immunoreactivity was observed in a perivascular location in the papillary dermis in seven of the AD cases (Fig. 1), and in two patients free nerve-fibre endings separated from blood vessels were seen. In skin from control subjects no SP-like immunoreactivity was found, except for one case where weakly stained single nerve fibres were observed in the superficial dermis.

Immunoreactivity to SOM was expressed around blood vessels in the superficial dermis of three control subjects, whereas no immunoreactivity was seen in AD patients. In six patients with AD and in one patient with prurigo, NPY-like immunoreactivity was observed within dendritic epidermal cells (Fig. 2). Such immunoreactivity was not detected in skin from normal control subjects, nor from the patient with generalized contact dermatitis. NPY-like immuno-

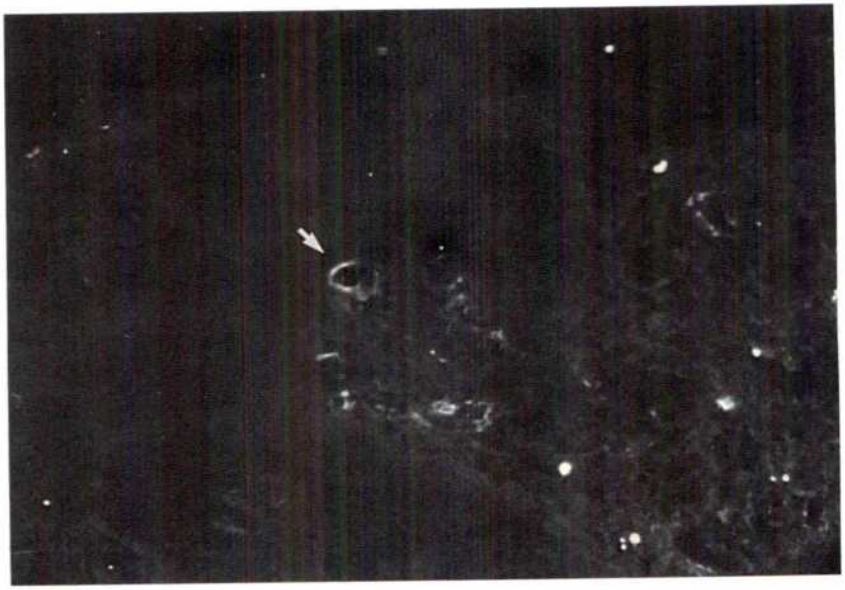

FIGURE 1. Immunofluorescent staining of lesional skin from an AD patient. SP-like immunoreactivity is expressed in a perivascular location in the papillary dermis (arrow) (× 250).

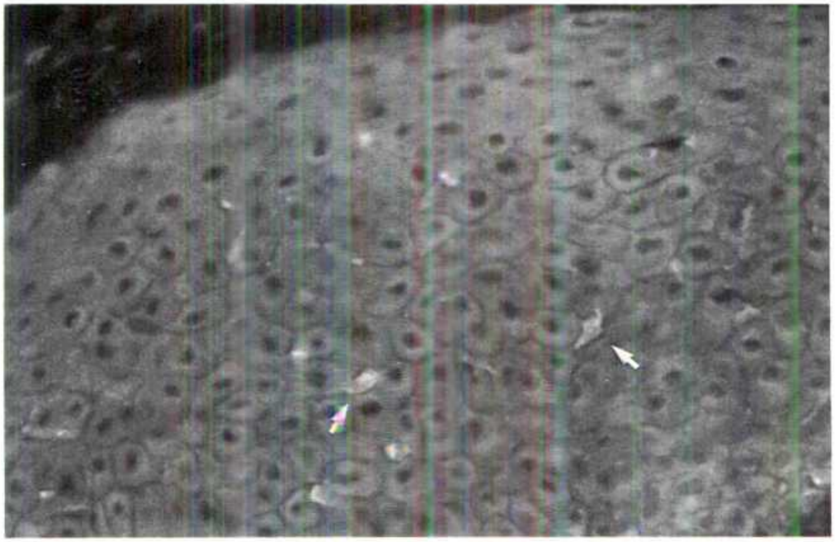

FIGURE 2. Immunofluorescent staining of lesional skin from an AD patient. NPY-immunoreactive dendritic cells are seen in the epidermis (arrow) (×400).

reactivity was only occasionally found around glands and blood vessels with no differences between AD patients and controls.

CGRP-like immunoreactivity was consistently observed as free endings in the papillary dermis close to the dermal–epidermal junction, with the same pattern and staining intensity in both AD patients and controls (Fig. 3). Similarly, VIP-positive nerve fibres were present

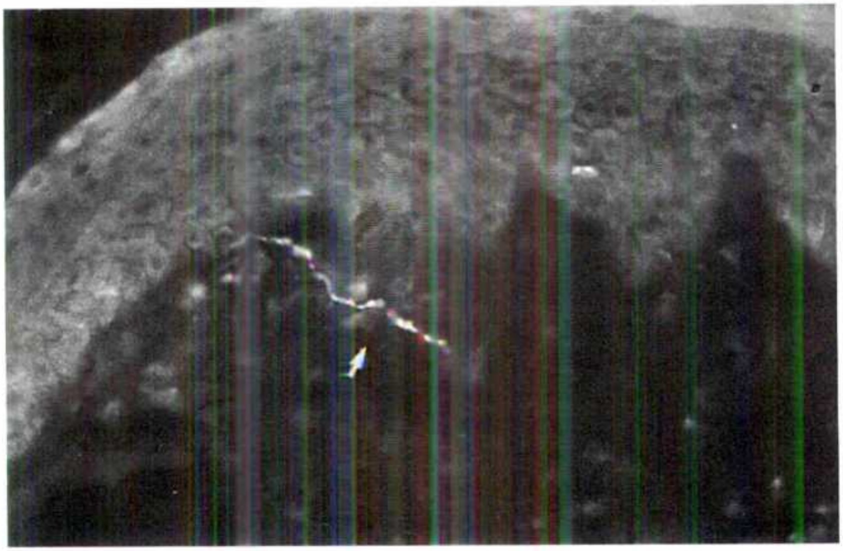

FIGURE 3. Immunofluorescent staining of skin from an AD patient showing a varicose CGRP-immunoreactive fibre in the papillary dermis (arrow) (×250).

around glands in both groups of patients. GAL-like immunoreactivity was not detected on nerve fibres, while in all sections a diffuse intracellular staining in the epidermis was observed. No clearly positive staining was found with NT or NKA antibodies in either group.

DISCUSSION

Substance P-positive nerves in lichenified patches of AD were markedly increased when compared with those in the normal controls. SP can act either directly on postcapillary venules to induce vasodilation and vascular permeability or indirectly by releasing vasoactive factors from mast cells.[1] Both these actions cause erythema and itching which are constant features of AD. Mast cells are predominantly located around the dermal blood vessels in human skin, and are frequently found to accumulate in AD.[15] SP-immunoreactive fibres are mostly found around the blood vessels in the papillary dermis, and several studies have now revealed that neuroeffector junctions exist between these mast cells and nerves in the skin.[16-18]

A slight increase of SP-immunoreactive nerves was observed in prurigo nodularis when compared with normal controls, while in neurodermatitis circumscripta there was no difference from healthy subjects.[19] In the same study CGRP-positive fibres were markedly increased in prurigo and unchanged in neurodermatitis circumscripta compared to controls. Although there are probably similarities in the pathogenetic mechanisms between these conditions and AD it is difficult to make any useful comparison. However, the unchanged expression of CGRP which we have found can be explained by the fact that its activity is regulated by SP,[20,21] which was more intensely expressed in the AD patients. The occurrence of SOM-immunoreactive fibres only in control subjects could be consistent with the concept that SOM inhibits the release of SP and therefore acts as a modulator of neurogenic inflammation.[22,23] Thus, in AD patients the absence of SOM-immunoreactive nerves may result in a lack of regulation of the mechanisms underlying neurogenic inflammation. SOM is also thought to be a physiological antagonist of SP in the immune system.[24] Since a 'disregulation' of immune functions is present in AD,[25] one could hypothesize a neuroimmunomodulatory role for SP and SOM in this disease.

Further evidence of possible interactions between some NP and the immune system is the observation that there is NPY-immunoreactivity on epidermal dendritic cells in AD patients. NP immunoreactivity has been observed by other authors on dendritic cells, e.g. SOM-like immunoreactivity has been shown on dendritic cells of both normal human skin and skin from patients with urticaria pigmentosa.[14,26] However, the significance of these findings is at present unknown.

While the intracellular staining pattern of GAL is probably due to a cross-reactivity, the absence of this NP in nervous structures could be related to the site of biopsy in the present study. So far, GAL-immunoreactive nerves have been reported only in fingertips and axillae.[27,28]

Our findings suggest a possible role of some NP in AD, although the results need to be verified in a larger group of patients. Further studies are in progress to investigate the relationships between NP and AD as well as other dermatoses where itching and erythema are characteristic features.

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A study of etretinate alopecia

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SUMMARY

Twenty-two patients receiving etretinate were studied in an attempt to clarify the changes in hair cycle dynamics responsible for etretinate alopecia. Shed hair counts and telogen counts on samples of plucked hair were performed prior to therapy and after 6 and 12 weeks' treatment with etretinate. No evidence of anagen effluvium was found. In two individuals there were findings compatible with a classical telogen effluvium, but these had atypical features. The main finding in the study was an increase in the plucked telogen count, maximal at 12 weeks. As an arrest in the onset of anagen could produce only a comparatively small telogen increase, the predominant cause of this increase was a decrease in the duration of anagen. In two subjects there was also evidence of a telogen anchorage defect.

Alopecia is a distressing side-effect of treatment with etretinate and may necessitate its withdrawal. Estimates of the incidence of this hair loss have varied widely from as low as zero[1] to as high as 76%.[2] It has been described as 'telogenic' in 70% and 'telogen dystrophic' in 22%.[3] The aim of this study was to investigate the hair cycle dynamics over the first 3 months of treatment with etretinate and to test the hypothesis that the hair loss is due to a telogen or anagen effluvium.

METHOD

Subjects

Twenty-two patients (20 female and two male), aged 21–76 years (mean 47 years) were investigated during treatment with etretinate. Eighteen were suffering from psoriasis, two from Darier's disease and two had discoid lupus erythematosus. The scalp investigation sites were not involved by any of these diseases. The patients were recruited over a 4-year period.

Treatment

The starting dose of etretinate was 25–75 mg daily, and in some this was adjusted during the study in accordance with the clinical response and side-effects, but not those that were related to the hair.

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Hair sampling

Investigations were performed immediately prior to starting etretinate and after 6 and 12 weeks of treatment. Five-day collections of shed hair were performed by the subjects, who were asked to wash their hair and then collect the shed hairs for the subsequent 5 days, without rewashing the hair. They were asked to groom the hair in a consistent manner and collect all the shed hairs found on the brush, comb, pillow and clothing. Plucked samples of an average of 50 hairs were taken using needle-holding forceps with the jaws wrapped with elastoplast to increase the grip. The sampling site was the vertex of the scalp.

Hair samples were coded and examined in a blinded manner. The shed hairs were counted and approximately 30 from each sample were arranged on a microscope slide and held in place with transparent adhesive tape over the shafts of the hairs. The proximal ends were examined

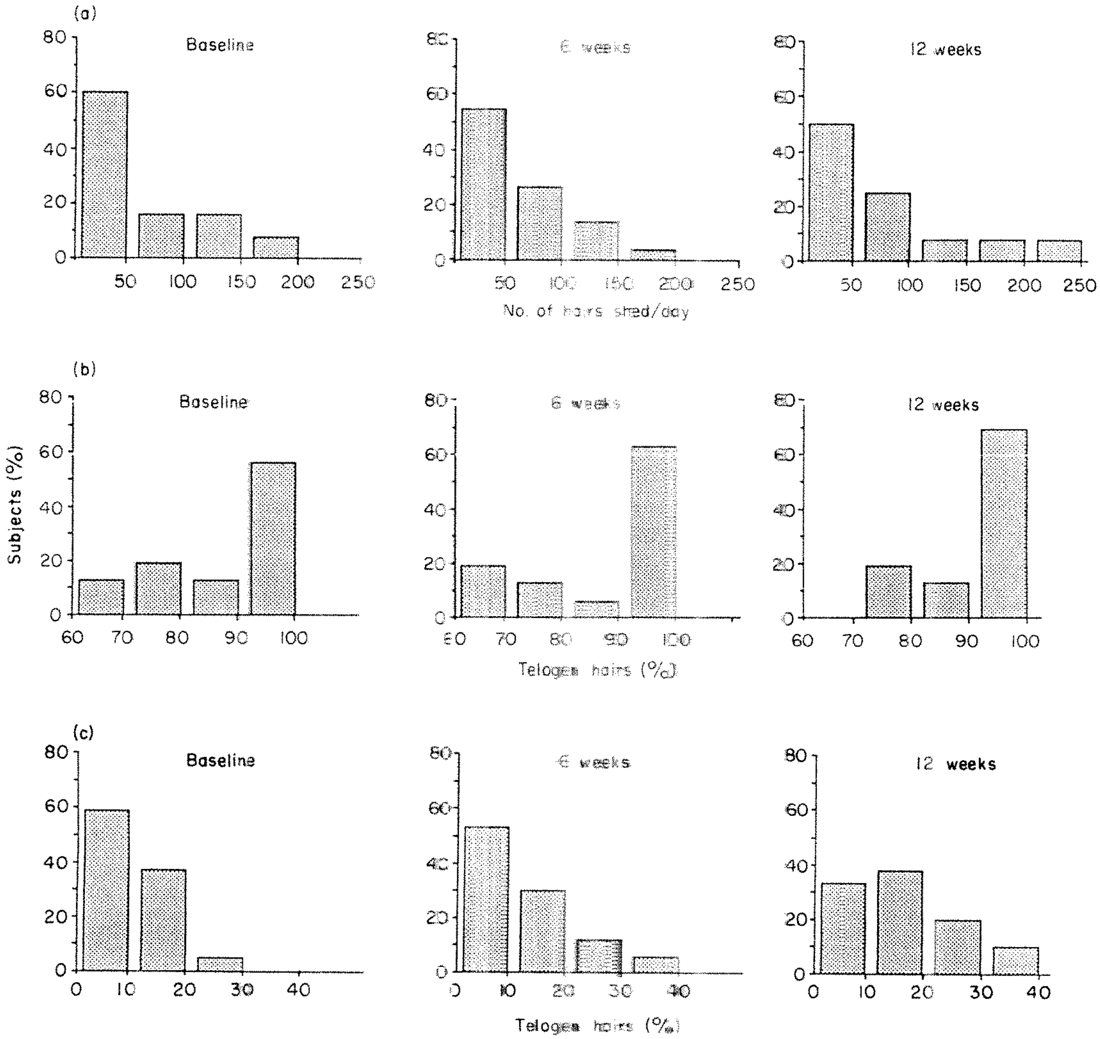

FIGURE 1. Summary of the data: (a) rate of loss of shed hair; (b) proportion of shed hair in telogen; (c) proportion of plucked hair in telogen.

microscopically and the proportions of telogen and anagen hairs determined. Fractured hairs were regarded as anagen hairs. The entire samples of plucked hairs were similarly examined.

Changes in shed hair counts were analysed using the Wilcoxon signed ranks matched pairs test. The changes in the proportions of telogen hairs were analysed by the Mantel-Haenszel test for a series of 2 × 2 contingency tables. To allow for possible heterogeneity of response, the variance of the test statistic was calculated using a permutation argument.[4]

RESULTS

The mean initial dose of etretinate was 52 mg (range 25–70 mg), the mean 6-week cumulative dose was 2,009 mg (range 1,050–3,150 mg) and the mean 3-month cumulative dose was 3,830 mg (range 2,520–6,300 mg).

Figure 1a illustrates the shedding rate throughout the study. The pretreatment mean was 57·5 hairs/day and in two individuals the rate was greater than 150 hairs/day. At 6 weeks following the onset of treatment the mean was 57 hairs/day and in one individual the rate was greater than 150 hairs/day. At 12 weeks of treatment the mean shedding rate was 72 hairs/day, which was not significantly different from the basal level ($P > 0{\cdot}05$). In four individuals the shedding rate was greater than 150 hairs/day and in two individuals greater than 200 hairs/day.

The proportion of shed hairs in telogen is displayed in Figure 1b. The mean percentage of hairs in telogen was 87% prior to treatment, 87% at 6 weeks of treatment and 91% at 3 months of treatment.

TABLE 1. The effect of a complete arrest of the onset of anagen on the plucked telogen count

	At onset of study	At 3 months of treatment
No. of hairs in telogen	Tc	Tc
No. of hairs leaving anagen → telogen in 3 months		$\frac{3\,Ac}{A}$
No. of hairs in anagen	Ac	$Ac-\frac{3\,Ac}{A}$
Plucked telogen count	$\frac{Tc}{Ac+Tc}$	$\frac{Tc}{Ac-\frac{3\,Ac}{A}+Tc}$
	$=\frac{T}{A+T}$	$=\frac{T}{A-3+T}$
(If $A=30$ months, $T=3$ months) Plucked telogen count	9%	10%

Assuming: (i) a normal continuing rate of shedding (as found in study); (ii) The number of hairs in anagen and telogen is proportional to the duration of anagen and telogen.

A = duration of anagen (months).
T = duration of telogen (months)
c = constant.

The results of plucked telogen counts are shown in Figure 1c. The mean telogen count prior to treatment was 9·5%. This rose to 14·1% after 6 weeks of treatment ($P > 0{\cdot}05$) and 15·9% at 12 weeks ($P < 0{\cdot}025$). The count exceeded 30% in no individuals prior to treatment, in one at 6 weeks, and in two at 12 weeks.

DISCUSSION

In no individual was there evidence of an anagen effluvium. In only two individuals were there findings compatible with a classical telogen effluvium, with a notable increase in hair shedding rate at 3 months and a significant increase in the plucked telogen count at 6 weeks. However, even in these two there were unusual features. In both, the telogen count remained elevated at 3 months despite the shedding, and in one the shedding rate was still elevated 6 weeks later, i.e. at 18 weeks following onset of treatment. The abnormalities in these patients therefore appear to have been progressive rather than abrupt and short-lived as in a classical telogen effluvium.

The hair cycle investigations in the present study have shown little or no change in shedding rate or shed hair type throughout the 3-month period. These results would appear to conflict with those of Mahrle *et al.*[3] who reported hair loss occurring at 5–8 weeks of treatment which was 'telogenic' in 70% and 'telogen dystrophic' in 22%. This difference is not a drug dosage effect as the mean cumulative dose at 5–8 weeks in Mahrle's study was 1·9 g and the mean cumulative dose at 6 weeks was 2g in this study.

The principal finding in this study was an increase in plucked telogen count at 3 months of 6·4% ($P < 0{\cdot}025\%$), and this was probably underway at 6 weeks. These levels are modest compared to those expected in the telogen effluvium induced by other drugs, such as heparin.[5]

The cause of this increase in plucked telogen count could be either a large change in a small number of individuals or a small change in a large proportion. Only two individuals developed a plucked telogen count of more than 30% at 3 months. If these individuals are discounted then the increase in telogen count at 3 months was still 4·2% above the basal level. Furthermore, the frequency distribution of plucked telogen counts at 3 months showed a shift of the mode to the right, with no obvious secondary peak to the right of the distribution. These results suggest that the plucked telogen count was affected in a substantial proportion, or possibly the whole population, of individuals.

There are two possible mechanisms for the raised telogen counts observed in this study: either a decrease in the duration of anagen or a delay in the onset of anagen. A decrease in the duration of anagen is the mechanism for the development of telogen effluvium, when approximately 30–40% of anagen hairs virtually simultaneously enter telogen. In only two patients were there results compatible with a classical telogen effluvium. In other individuals the lack of shedding at 3 months and the lateness of the maximal telogen increase, occurring at 3 months, argue against this mechanism. In a classical telogen effluvium, there is an abruptness in the alteration of the duration of anagen and therefore there is inevitably a wide variation between affected follicles in the duration of anagen. An alternative mechanism, compatible with the present findings, is an ongoing decrease in the duration of anagen of a lesser degree than that found in a classical telogen effluvium and of a more similar degree between hair follicles. Thus, the telogen count would steadily increase over a number of months, as was found in this study, and the shedding rate would increase later. Prolonged observation of shedding rate and plucked telogen counts after 3 months would provide further evidence of such a mechanism.

Another possible mechanism for the increase in plucked telogen level at 3 months is a delay in the onset of anagen where the replacement of hairs lost as part of normal telogen shedding would be defective. Hence, there would be a relative increase in plucked telogen count. An observable hair effect as early as 5–8 weeks on treatment would be noted,[3] as would a persisting decrease in hair density throughout the treatment period which is commonly observed. Conversely, an exclusive decrease in the duration of anagen could explain neither of these clinical observations. However, even a complete anagen onset arrest could only produce a 1% increase in plucked telogen count over a 3-month period, so this mechanism alone cannot account for the observed increase in plucked telogen count (Table 1). Whilst a contribution from this mechanism is not precluded, shortening of the duration of the anagen must be the principal mechanism.

There was a further interesting finding in two patients in the study. These were the only two to show a shedding rate of greater than 200 hairs at 3 months. In one, significant shedding started before 6 weeks of treatment and in the other there was no associated increase in plucked telogen count. This suggests that there is a decreased hair-holding capacity of the telogen follicle.

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Recurrent papillomas of the nipple associated with human papillomavirus 41

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SUMMARY

A case is described of a man with multiple papillomas on the nipple that resembled clinically and histologically condylomata acuminata. Human papillomavirus (HPV) type 41 DNA sequences were detected in the biopsy material. There was no evidence of any immunodeficiency.

Fifty-seven distinct types of human papillomaviruses (HPV) have been identified and some cause benign epithelial or epidermal tumours such as common warts[1] and condylomata acuminata.[2,3] Other HPV types 16, 18, 31, 33 and 35[4–8] have been closely linked to genital cancers. Patients with epidermodysplasia verruciformis (EV) HPV types 5, 8, 14 and 17 have been related both to benign lesions and to squamous cell carcinomas.[9–14] Recently a new type, HPV 41, was identified in a patient with multiple lesions that resembled common warts. In screening 100 benign and malignant skin lesions, HPV 41-related DNA sequences were found in two of 10 squamous cell carcinomas and in one of three keratoses due to arsenic.[15]

We report the identification of DNA sequences of HPV 41 in recurrent benign papillomas of the nipple, and the detection of viral core antigens in these lesions.

PATIENT AND METHODS

Patient

A 44-year-old Caucasian male developed multiple papillomas of the right nipple. These resembled condylomata acuminata and were up to 2 mm in diameter (Fig. 1). Despite surgery and cryotherapy the lesions recurred over a period of 5 years. There was no evidence of any other past or present HPV infections. To exclude an immunodeficiency the patient was challenged with a number of recall antigens (Multitest Meriux®) and had a normal score. The lymphocyte

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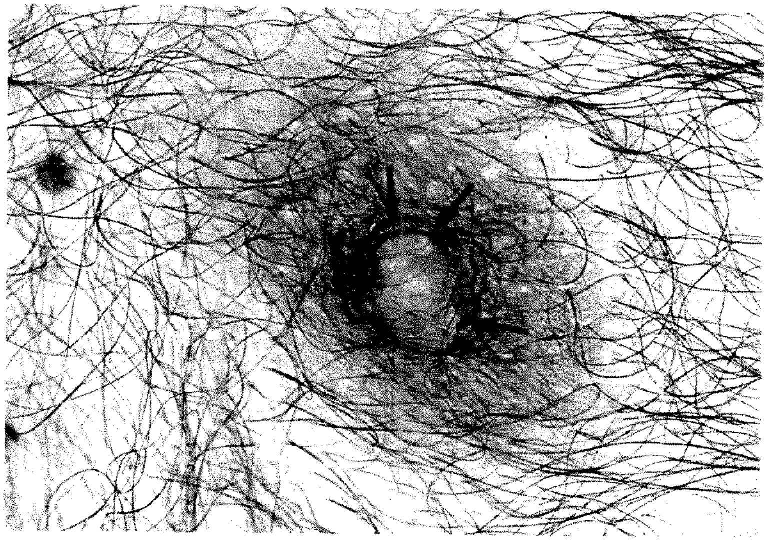

FIGURE 1. Small papillomas with slight hyperkeratosis at the right nipple (arrow).

subsets were within the normal range, $CD4^+$ 1·3/nl, $CD8^+$ 0·9/nl, B cells 0·2/nl and NK cells 0·8/nl.

HPV typing

After excision the papilloma was divided into three parts. One part was snap-frozen in liquid nitrogen and kept for the extraction of high molecular weight DNA. The method of reverse blot[16] was used to identify the HPV present in the lesion. The clones of HPV types 1–38 and 41

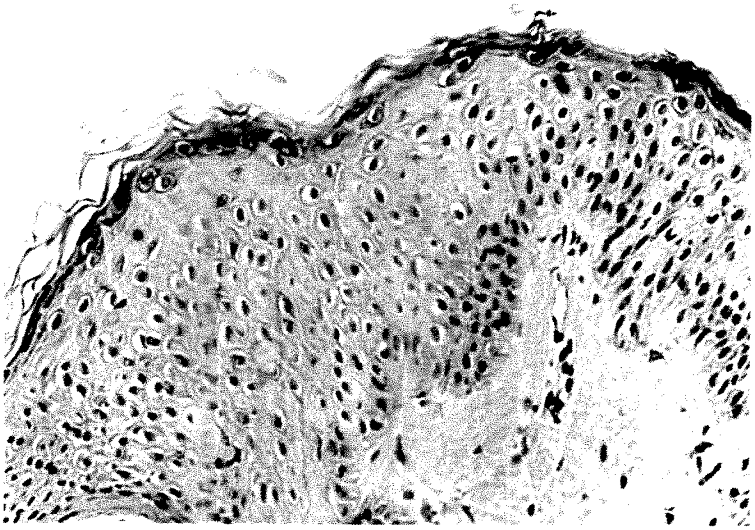

FIGURE 2. Histology of papilloma showing orthokeratosis and acanthosis with koilocytes in focal distribution (haematoxylin and eosin).

were each digested with its respective restriction enzyme used for cloning. The viral DNA was then separated from the vector DNA by gel electrophoresis, after which the DNA was transferred to a nitrocellulose filter. This blot was hybridized to radioactively labelled total cellular DNA using conditions of high stringency (T_m −20°C). Subsequently, unlabelled cellular DNA was digested with the restriction enzyme Eco R1 and, after gel electrophoresis, transferred to a nitrocellulose filter. This Southern blot was hybridized (T_m −20°C) with ^{32}P-labelled HPV 41 DNA.

The second portion of the biopsy was fixed in formaldehyde saline, embedded in paraffin, and sections cut and stained with haematoxylin and eosin. Sections were also cut and investigated for group-specific papillomavirus antigens using a peroxidase-antiperoxidase (PAP) technique. An antiserum to bovine papillomavirus type 1 (BPV 1) (Dako, Santa Barbara, CA, U.S.A.) was used and the procedure was the same as previously described,[17] other than the antiserum was diluted 1:500 with Tris-buffer.

The third part of the biopsy was fixed in glutaraldehyde and osmium-tetroxide and later embedded in Epon® for electron microscopy. Paraffin-embedded material was also used and, after being deparaffinized, was re-embedded in Epon® for electron microscopy.

RESULTS

The histology showed orthokeratosis with marked acanthosis and papillomatosis of the epidermis. There were large keratinocytes with perinuclear vacuolization and small hyperchromatic nuclei in a focal distribution throughout the epidermis (Fig. 2) and the histology resembled that of genital condylomata acuminata.

On electron microscopy, there was perinuclear cytoplasmic oedema but no papilloma virions were found (Fig. 3). Immunohistological examination with antibodies raised against BPV 1 disclosed a few cells with nuclei staining strongly for papillomavirus common antigens (Fig. 4).

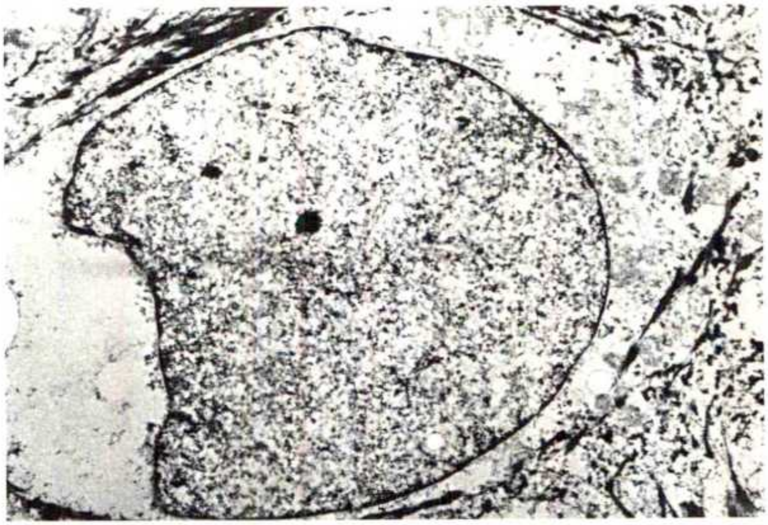

FIGURE 3. Electron micrograph showing perinuclear cytoplasmic oedema of a stratum spinosum cell. No virions are visible in the nucleus (× 10,300).

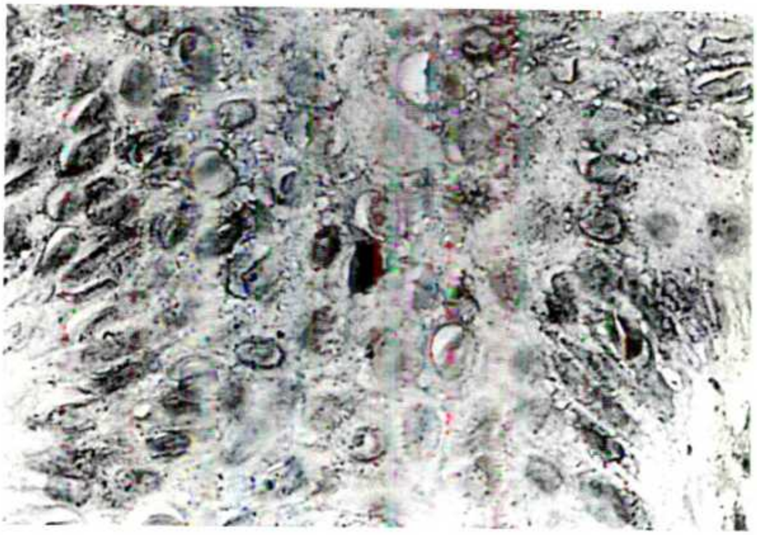

FIGURE 4. Immunohistochemical peroxidase-antiperoxidase staining of papillomavirus common antigens in a keratinocyte nucleus.

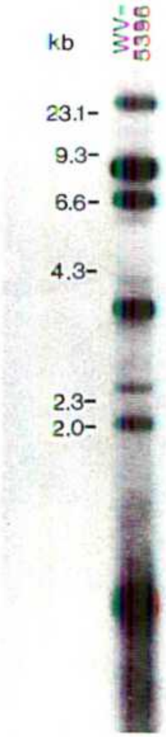

FIGURE 5. Southern blot autoradiograph of cellular DNA (after Eco R1 digestion) from a papilloma of the nipple hybridized under stringent conditions to ^{32}P-labelled HPV 41 DNA.

Under conditions of high stringency, DNA isolated from the biopsy cross-hybridized with HPV 41 DNA only. To confirm these results, a Southern blot of the cellular DNA was hybridized with ^{32}P-labelled HPV 41 DNA. The autoradiograph (Fig. 5) showed positive bands of varying intensity. The expected fragment sizes of HPV 41 obtained after digestion with Eco R1 would be 5·07 kb, 2·2 kb, 0·37 kb and 0·06 kb. As the cross-hybridizing fragment sizes obtained differed, either HPV 41 DNA was integrated into the cellular DNA or a virus closely related, but not identical, to HPV 41 was identified. As no more DNA was available, no further classification was possible.

DISCUSSION

Human papillomaviruses associated with extra-genital squamous cell carcinomas have been found in patients suffering from EV, a rare chronic disease characterized by disseminated warts[18] in which a depressed cell-mediated immunity is often found. These carcinomas have been shown to contain HPV 5 or 8 DNA and, in single cases, HPV 14 or HPV 17 DNA.[9-14] These types of HPV are also to be found in patients on immunosuppressive therapy such as renal allograft recipients.[19-22] However, these HPV types have not yet been reported in immunocompetent patients.

HPV 41 was isolated from the benign common wart-like lesions of a 15-year-old girl without clinical signs of EV.[15] She suffered from chronic atopic dermatitis, a disease known to be associated with decreased cellular immunity.[23] Subsequently, HPV 41 DNA has been detected in some cases of squamous cell carcinoma and in a patient with arsenical keratoses,[15] a well-recognized precursor of squamous cell carcinoma.[24] These may indicate an oncogenic potential for HPV 41.

In the present case, HPV 41 DNA was identified in benign papillomatous lesions of the nipple that resembled clinically and histologically condylomata acuminata. Viral replication could be demonstrated within these lesions. The patient had no history of immunosuppression nor any evidence of immunodeficiency.

It is of importance that the type of HPV, the DNA of which was identified in cases of squamous cell carcinomas, was also demonstrated in benign lesions of an immunocompetent patient. Further screening of unusual warts and squamous cell carcinomas and their precursor lesions are required to determine the role of HPV 41 in the pathogenesis of skin cancer.

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Hidradenitis suppurativa: a disease of follicular epithelium, rather than apocrine glands

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SUMMARY

A retrospective study was made of the histopathology of axillary skin excised fom 12 patients with hidradenitis suppurativa. In the majority of cases (10 out of 12), squamous epithelium-lined structures, in the form of cysts or sinuses were identified in the dermis. Laminated keratin was present in all these structures and half of the cysts also contained hair shafts, suggesting that they are derived from hair follicles. Only a small proportion of cases (4 out of 12) displayed inflammation in apocrine glands, and in these the inflammation was also seen around eccrine glands, hair follicles and the epithelium-lined structures. However, in cases where the epithelium of the 'cysts' was disrupted, the inflammatory infiltrate appeared to be centred around these areas. These observations suggest that the squamous epithelium-lined structures, which probably represent abnormal dilated hair follicles, are a more constant diagnostic feature in hidradenitis suppurativa than inflammation of apocrine glands which appears to be a secondary phenomenon.

Hidradenitis suppurativa was first described as a distinct entity by Velpeau[1] in 1839. He reported a patient with an inflammatory process involving the skin of the axillary, mammary and perianal areas. Verneuil,[2] in 1854, described a series of patients with similar lesions and was the first to suggest that they were abscesses of the sweat glands. However, it was not until 1922 that hidradenitis became associated with the apocrine glands.[3] A detailed description of the histology in hidradenitis suppurativa was made by Brunsting.[4] He observed that the earliest cellular reaction was in the subcutis, primarily within the lumen of the apocrine glands and in the neighbouring peri-glandular connective tissue, and that many of the apocrine glands had their lumen distended with leucocytes.

The pathogenesis of this condition is still unknown, and there is a lack of evidence to support the original concept of hidradenitis suppurativa being primarily a disease of apocrine glands. No

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significant differences have been found in the size and density of the aprocrine glands in hidradenitis suppurativa compared with normal controls.[5] It has been postulated that the initiating event in the disease is occlusion of the apocrine duct orifice. Some support for this comes from keratinous plugging of the apocrine duct produced in the axillae of 12 normal male subjects by the application of perforated belladonna adhesive tape to manually depilated axillary skin.[6] Three of the 12 subjects developed lesions at the tape sites which clinically resembled hidradenitis suppurativa. However, this study may not represent an appropriate model for the disease.

Hidradenitis suppurativa may also be seen in association with other conditions where occlusion of follicular structures plays a more obvious role, namely acne conglobata and dissecting folliculitis of the scalp. These three conditions have been referred to collectively as the 'follicular occlusion triad'. More recently another component, pilonidal sinus, has been added to form a 'tetrad'.[7]

In order to gain more information about the pathogenesis of hidradenitis suppurativa, a retrospective study was made of the histology in axillary skin from patients with this condition.

METHODS

Specimens of axillary skin surgically excised from 12 patients with clinically established hidradenitis suppurativa were examined. The tissue was fixed in 10% formol saline and processed routinely. Serial sections 4-μm in thickness were cut and stained with haematoxylin and eosin. The histological features were compared with control specimens of post-mortem axillary skin obtained from 10 cadavers with no previous history of hidradenitis suppurativa or other dermatological problems and with axillary skin surgically excised from one patient with hyperhidrosis. Particular attention was paid to the type and distribution of inflammatory changes in the skin, and to morphological abnormalities of the sweat glands and the pilo-sebaceous units. In cases where the inflammatory infiltrate was dense and the sweat glands difficult to recognize on haematoxylin and eosin-stained sections, an immunoperoxidase stain for cytokeratin CAM 5.2 was used. This delineates the sweat glands clearly from the surrounding structures, and the eccrine glands show a more intense and uniform staining than the apocrine glands, providing an additional means of distinguishing them, even in the presence of severe inflammation.

RESULTS

In specimens from 10 out of 12 patients with hidradenitis suppurativa, cystic structures lined by stratified squamous epithelium and containing laminated keratin were identified in the dermis (Fig. 1). Half of these contained free hair shafts, suggesting that they represent abnormal dilated hair follicles (Fig. 2). In four cases, there were also epithelium-lined sinus tracts extending directly from the surface epidermis into the deep dermis. Axillary skin from one of the patients contained a cyst lined by squamous epithelium with some adjacent eccrine glands but no apocrine glands. In all the specimens, the apocrine glands showed no significant morphological abnormalities.

Skin from all the patients exhibited chronic inflammatory changes with extensive scarring in the dermis and subcutis. Acute inflammation was also present in eight of the 12 cases. However, in specimens from seven patients, there was no inflammation in apocrine glands, despite inflammatory cells being identified around hair follicles and the epithelium-lined cysts and

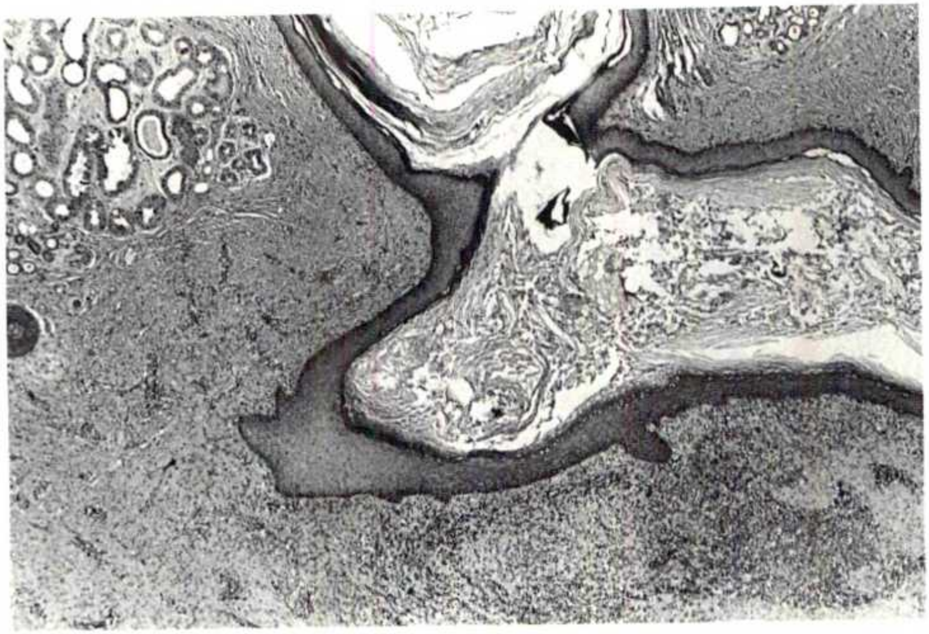

FIGURE 1. Axillary skin showing irregular epithelium-lined structure in a case of hidradenitis suppurativa. The inflammatory cell infiltrate is most intense adjacent to the squamous epithelium (bottom of picture) and peri-follicular scarring is prominent (particularly on the left). The apocrine sweat glands appear normal (top left and right). (H & E × 32)

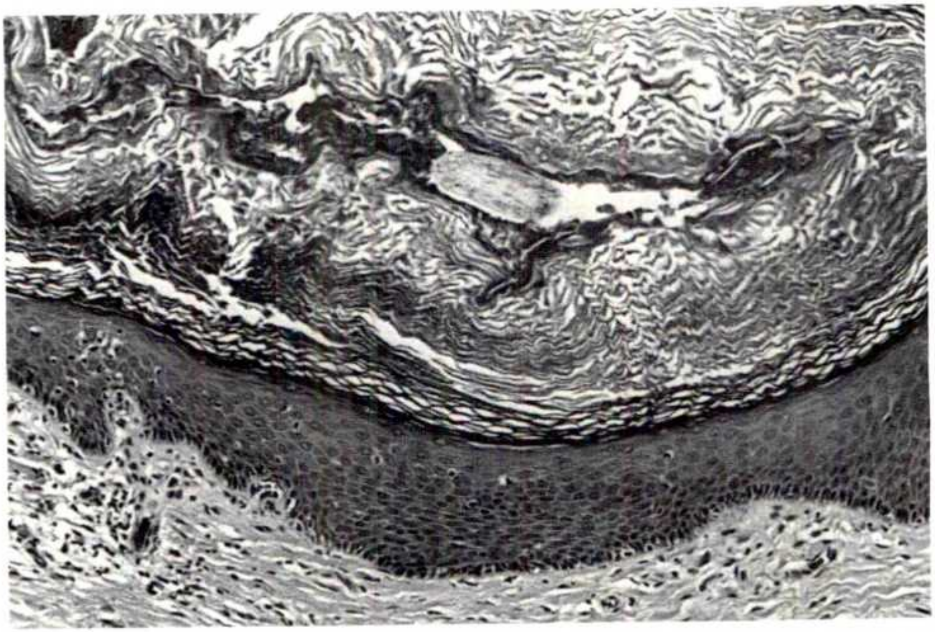

FIGURE 2. Laminated keratin and hair shaft seen within one of the cystic structures in axillary skin. (H & E × 125).

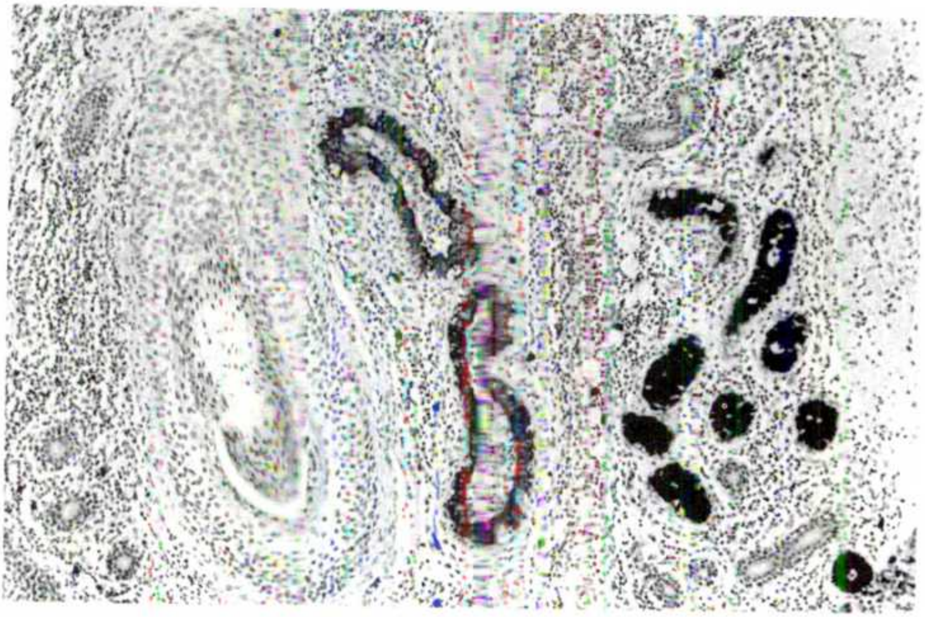

FIGURE 3. A case with extensive inflammation involving not only the apocrine glands (in the centre of the field) but also eccrine glands (on the right) and a hair follicle (to the left). (CAM 5.2 × 80).

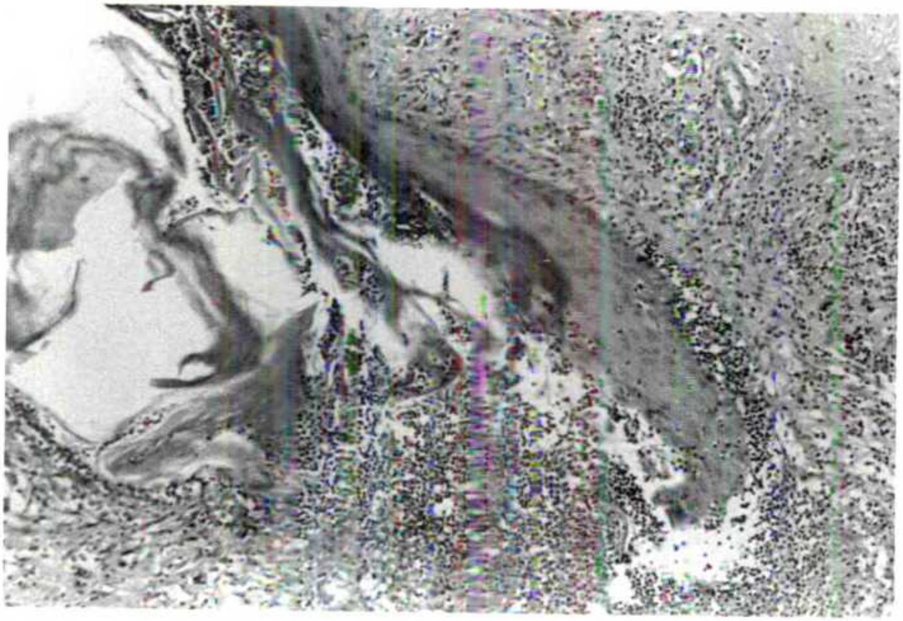

FIGURE 4. A dense infiltrate of neutrophils around the disrupted follicular epithelium (H & E × 80).

TABLE 1. Histological features in hidradenitis suppurativa (total number of patients = 12)

Feature	No. patients affected
Cystic epithelium-lined structures (5/10 with disruption of epithelium)	10
Sinus tracts lined by squamous epithelium	4
Chronic inflammation and scarring	12
Acute inflammation	8
No inflammation in apocrine glands	7
Extensive inflammation (involving all dermal and appendageal elements)	4

tracts described above (Table 1). In four cases, inflammatory changes did involve apocrine glands, but in these the inflammation was extensive and was also present around other structures, including eccrine glands, hair follicles and the cystic structures (Fig. 3). None of these cases showed inflammation centred preferentially around the apocrine glands. In contrast, five of the 10 cases with 'cysts' displayed discontinuity of the squamous epithelium lining these structures, suggesting ulceration or rupture, and in these sections, the inflammatory infiltrate was most intense around the disrupted epithelium (Fig. 4).

DISCUSSION

The histopathological findings suggest that squamous epithelium-lined structures, taking the form of cysts or sinuses, are a more consistent diagnostic feature of hidradenitis suppurativa (being present in 10 out of 12 cases) than inflammation of apocrine glands (found in only four out of 12 cases). The presence of hair shafts within these structures supports the view that they are abnormal dilated hair follicles. This is in agreement with the view that hidradenitis suppurativa is a disease of 'follicular occlusion', rather than a primary 'apocrinitis'. The inflammation seen in these cases did not seem to involve the sweat glands more than other neighbouring structures. However, where the epithelium-lined structures showed evidence of rupture, the inflammatory cells did appear to be most densely distributed at these sites. A review article on hidradenitis suppurativa stated that dilatation of the apocrine gland was a feature and that this eventually led to rupture of the gland with spread of the infection to adjacent apocrine and eccrine glands.[8] In no case were we able to demonstrate gross dilatation of the apocrine glands or any discontinuity of the cells lining the glands or their ducts. The ruptured cysts in our cases had a lining of stratified squamous epithelium similar to the epidermis and the epithelium lining hair follicles.

Although the work of Shelley and Cahn[6] mentioned above has frequently been quoted in the literature as an experimental model for hidradenitis suppurativa, it may not be an accurate model for a number of reasons. Manual depilation of the skin is a traumatic procedure and may produce changes in hair follicles and surrounding tissues, in the same way that shaving skin with a safety razor has been shown to predispose to infection.[9] The addition of atropine to the tape which was applied to the axillae of the subjects cannot be regarded as simulating a physiological process. Also, lesions clinically resembling hidradenitis suppurativa were produced in only 25% of the patients. Other investigators[10] have pointed out that the acute lesions induced have not progressed to the chronic condition recognized as hidradenitis suppurativa. Histological examination of axillary skin from the three positive cases of Shelley and Cahn showed keratinous plugging of dilated apocrine sweat ducts and inflammatory changes confined to a single apocrine sweat gland unit. The adjacent glands, hair follicles and eccrine glands appeared entirely normal. We have not seen this, even at the edge of the inflammatory lesions bordering on normal skin, which might have been expected to show the early changes of hidradenitis suppurativa.

Further evidence that occlusion of the apocrine duct orifice is not involved in the pathogenesis of hidradenitis suppurativa comes from comparison with Fox-Fordyce disease. This condition presents as a chronic papular eruption associated with pruritus, in areas containing apocrine glands. Histological examination of skin from these patients shows follicular plugging in the epidermis with occlusion of the orifice of the apocrine ducts. The continued secretion of sweat leads to rupture of the duct and the formation of aprocrine sweat retention cysts.[11] The follicular plugging in this condition produces specific changes in the apocrine glands, yet the clinical and pathological features of this disease are distinct from those of hidradenitis suppurativa.

The pathological features of hidradenitis suppurativa that we have observed bear many similarities to those of acne. The epithelium-lined structures resemble comedones.[12] In a review of a large series,[13] 70% of patients with hidradenitis had active acne or evidence of past acne. Hidradenitis suppurativa is distinguished by its peculiar distribution, and by chronic relapsing inflammation, which is difficult to manage both medically and surgically. The predilection of this disease for particular anatomical sites corresponds not only to the distribution of apocrine glands in the body, but also to the distribution of terminal hair follicles dependent on low concentrations of androgens. Hidradenitis may be considered as a suppurative inflammation of these terminal hair follicles, with their associated apocrine, eccrine and sebaceous glands being secondarily involved. Acne is a disease of sebaceous follicles, but the fundamental change in both conditions may be similar, i.e. hyperkeratosis of follicular epithelium leading to formation of comedo-like structures. The bacteriology of acne is characteristic, *Propionibacterium acnes* being found in virtually all comedones,[12] and there is evidence for this micro-organism being the mediator of comedo rupture, which leads to inflammation. In hidradenitis suppurativa, a wide variety of different bacteria have been isolated,[14] but not *P. acnes*. Treatments in acne vulgaris have not been uniformly effective in hidradenitis suppurativa and antibiotics do not produce any long-term remission. The results of treatment with isotretinoin have at best been equivocal, although therapy with the anti-androgen cyproterone acetate appears to be more successful.[15,16]

In our histological review, inflammation of the apocrine glands does not appear to be a primary feature of hidradenitis suppurativa, or a useful diagnostic feature. These findings agree with those of Plewig and Steger,[17] who have used the term ‘acne inversa’ to encompass hidradenitis suppurativa, as well as the other diseases in the follicular occlusion triad and tetrad. This concept takes into account the similarities, as well as the differences, between these conditions and acne.

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Elucidation of chemical compounds responsible for foot malodour

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SUMMARY

Short-chain fatty acids from the socks and feet of subjects either with strong foot odour or with weak or no foot odour were extracted with ethyl ether, and then analysed by gas chromatography/mass spectrometry (GC/MS). Short chain fatty acids were found in greater amounts from those subjects with strong foot odour. Iso-valeric acid was present in all the subjects with foot odour but was not detected in those without. Olfactory evaluations of the various short-chain fatty acid solutions were in agreement with the GC/MS analyses. By incubating sweat and lipid from subjects with strong foot odour, we succeeded in reproducing the foot malodour. GC/MS analyses of reproduced foot odour revealed that short-chain fatty acids were present in a similar composition to that found *in vivo*.

It is generally accepted that offensive body odours are generated by the decomposition by micro-organisms of organic substances derived from the eccrine, sebaceous and apocrine glands, into volatile compounds of low molecular weight. Each region of the body appears to have its own key odour that gives the site a distinctive smell. Studies involving the sampling and identification of volatile compounds from the body and from different sites, most of which were performed by means of gas chromatography/mass spectrometry (GC/MS), have been reviewed.[1,2] Although a number of compounds including short-chain fatty acids, nitrogen-containing compounds and sulphur-containing compounds, etc. have been identified within body odour, few reports have suggested a key odour for a certain body site.

Products aimed at reducing or eliminating offensive body odours are available and most contain either aluminium salts to inhibit perspiration or antimicrobials to inhibit odour-forming bacteria. Masking agents such as perfumes are often included, but these are not always satisfactory. Our aim is to develop a deodorant that acts directly on the chemical compounds responsible for offensive body odours. In this study we have concentrated on analysing those compounds responsible for foot odour and, as short-chain fatty acids appeared to contribute, we have investigated their relationship with foot malodour.

METHODS

Olfactory evaluation of short-chain fatty acid aqueous solutions

A total of eight aqueous solutions were prepared, each containing 1000 ppm of short-chain fatty acids (C2–9). Each solution was put in a numbered glass bottle. Our panel of 'sniffers' consisted of 10 men and 10 women aged 20–35 years who had not been specially trained to detect the compounds under study. With each of the eight solutions, after sniffing, the subjects were asked if they could recognize any smell and those who could were asked whether it resembled foot or axillary odour or neither. The same assessments were carried out with different concentrations of solutions containing 100, 10, 1 and 0·1 ppm.

Sampling of foot odour

Five healthy male subjects aged 25–30 years with strong foot odour, and five healthy males of the same age who considered that they had little or no foot odour at all, were also chosen. All the subjects wore pre-ether-extracted socks (synthetic fibre) and performed physical exercise by means of an ergometer (30 rpm) under 35°C at 90% humidity for 30 min. After the physical exercise, five of the subjects had a strong foot odour whilst the other five had either a faint or no odour at all to their feet. Extracts from the socks were obtained using ethyl ether with a Soxhlet's extractor for 5 h. Extracts from the feet were obtained using ethyl ether which was evaporated in a vacuum and the ether-free extract dried and placed in a micro-reaction vial. To the extract was added 2 ml of 0·5% p-toluene sulfonic acid methanol solution. The micro-reaction vial was placed inside a 100°C oven for 60 min for methyl esterification and the sample then analysed by GC/MS.

Reproduction of foot malodour

Attempts were made to reproduce the malodour using sweat and lipid. The subjects who took part in the study of *in vivo* foot odour wore a sterilized plastic bag around the body so that sweat would pour into the bag during physical exercise. Following exercise the lipids were extracted from their backs using acetone as the solvent. The lipids extracted from both the foot and back were almost identical, as determined by thin layer chromatography (TLC). The sweat sample was placed inside a 100-ml Erlenmeyer flask, where it was concentrated in a vacuum to approximately 7 ml. After evaporating the acetone in a vacuum, 3·5 mg of the lipid was added to the concentrated sweat. The flasks were placed inside a 37°C oven and left for 10 days. The samples were analysed by GC/MS but, prior to extraction with ether, hydrochloric acid (6 M) was added to adjust the pH to 1 so that the short-chain fatty acids would be efficiently extracted from the water-rich matrix into the ether layer.

GC/MS analysis

All the foot and reproduced foot malodour samples were introduced into a Hitachi M-80 mass spectrometer via a Hewlett Packard 5710A gas chromatograph. The gas chromatograph was equipped with a Hewlett Packard capillary column (Ultra 2, 5% phenylmethylsilicone, 0·52 μm, 0·31 mm id, 25 m). The column temperature was programmed from 10 to 320°C at a rate of 5°C/min. The injector temperature was maintained at 320°C and 0·5 μl of each sample was injected in the splitless mode. The mass spectrometer was operated at full accelerating voltage (3 kV) scanning a mass range of 0 to 500 a.m.u. EI (electron impact) mass spectra were acquired at an ion source temperature and electron multiplier voltage of 200°C and 2 kV, respectively. Mass chromatograms were monitored at mass number (m/z) 74, a characteristic ion observed for fatty acid methyl esters due to McLafferty rearrangement. For identification of the observed peaks, mass spectra and GC retention times were compared to those of authentic short-chain fatty acid methyl esters.

RESULTS

Olfactory evaluation of short-chain fatty acid aqueous solutions

The assessors responded positively to all the 1000 ppm solutions. Some were not able to detect an odour from the diluted solutions. The results for the 1000 ppm solutions (Fig. 1) show that each fatty acid was considered to resemble either foot or axillary odour by at least two assessors. Iso-valeric acid (iso C5) was considered to resemble foot odour by half of the assessors, and acetic (C2) and butyric (C4) acid by six of the 20 assessors. Valeric acid (C5) and enanthic acid (C7) were considered to resemble axillary odour most, but in each case by only four out of 20. We can assume from this result that foot odour is more distinctive than axillary odour in terms of its relation with short-chain fatty acids. Short-chain fatty acids considered to resemble axillary odour tended to be higher in carbon number than those that resembled foot odour. Iso-valeric acid was considered to have the odour of feet at all concentrations. Some assessors, although evaluating iso-valeric acid as not resembling foot odour at 1000 ppm, found it to resemble foot odour at diluted solutions. A total of 70% of the assessors found iso-valeric acid at least at one concentration to resemble foot odour.

Analysis of foot odour

Figure 2 compares the mass chromatograms obtained from analysing the extract from the sock of a subject with strong foot odour and that from a subject with hardly any odour. The latter half of the chromatogram is the region where fatty acid methyl esters of comparatively high molecular weight elute. The peaks eluted are fatty acids derived from lipids and they are more or less identical. However, in the first half of the chromatogram, considerable amounts of short-chain fatty acids are detected from the subject with foot odour (Fig. 2b), whereas hardly any occur in the subject with no foot odour (Fig. 2a). The sock extract samples analysed from subjects with foot odour all exhibited more short-chain fatty acids than those from subjects

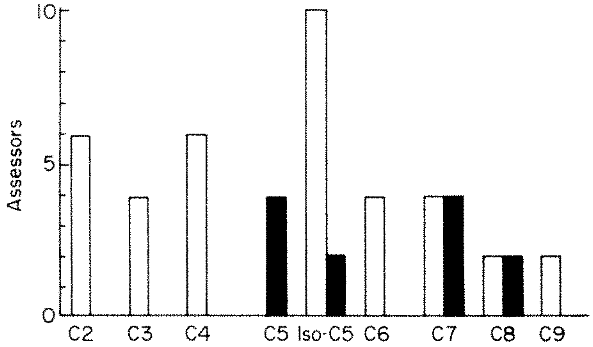

FIGURE 1. Olfactory evaluation of short-chain fatty acid solutions (1000 ppm). The vertical scale shows the number of assessors who responded to each corresponding acid as resembling either foot (□) or axillary (■) odour.

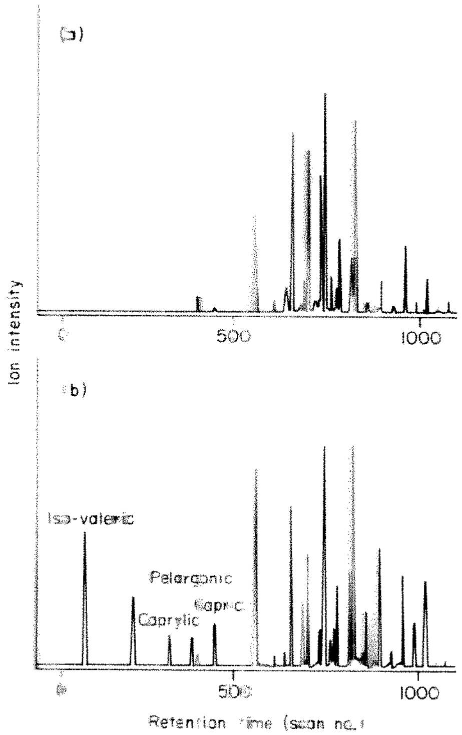

FIGURE 2. GC/MS analysis of sock extract. Mass chromatograms from (a) a subject without foot odour and (b) a subject with foot odour.

without foot odour; this also applied to foot extract sample (Fig. 3). Iso-valeric acid was abundant in all the subjects with foot odour but was never detected from those without foot odour.

Reproduction of foot malodours

The odour reproduced from the sweat and lipid of subjects with strong foot odour resembled that found *in vivo*. The intensity of the odour increased and reached a maximum by the tenth day after incubation. Only a faint odour was generated by incubating the sweat and lipid of subjects without foot odour. GC/MS analysis of reproduced foot odours resembling actual foot odour revealed short-chain fatty acids including iso-valeric acid, and their compositions were found to be similar to *in vivo* foot odour samples.

Whilst analysing the faint or almost odourless samples generated by incubating the sweat and lipid from subjects with weak or no foot odour, an interesting phenomenon was observed. Upon addition of hydrochloric acid for pH adjustment, a pungent odour resembling that of strong foot odour was suddenly generated. When these samples were analysed by GC/MS, short-chain fatty acids including iso-valeric acid were identified (Fig. 4).

DISCUSSION

The smell of iso-valeric acid was found to resemble most clearly that of foot odour, as determined by 14 of our 20 assessors. Iso-valeric acid has been considered as the primary

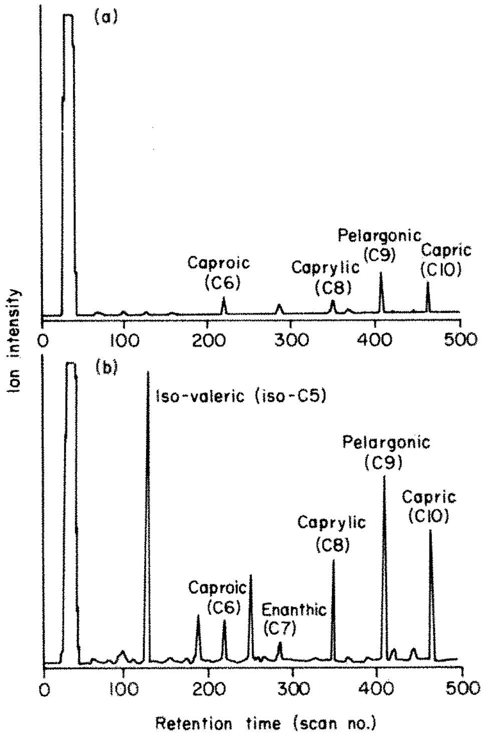

FIGURE 3. GC/MS analysis of foot extract. Mass chromatograms from (a) a subject without foot odour and (b) a subject with foot odour.

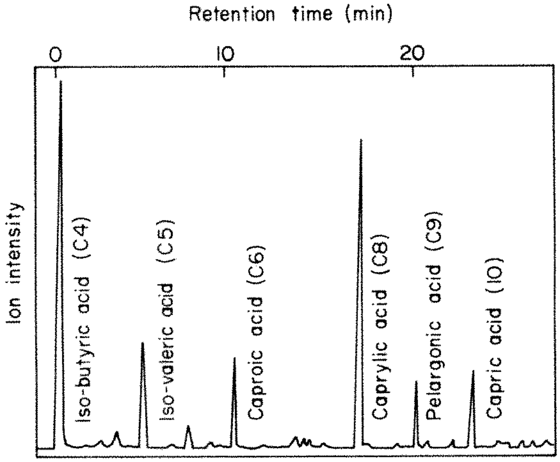

FIGURE 4. GC/MS analysis of reproduced foot odour. The sample was from a subject without foot odour and was originally odourless, but generated strong foot odour upon addition of hydrochloric acid.

chemical odorant of sweat, being one of six primary human odours.[3] Specific anosmia of iso-valeric acid was reported to be 3%, the lowest among the six primary odorants, this fatty acid having a distinct odour recognizable by most people. All of our assessors could recognize iso-valeric acid at a concentration of 100 ppm.

GC/MS analysis revealed that short-chain fatty acids were responsible for foot odour, as they were detected in excess from subjects with strong foot odour compared to those without. Iso-valeric acid was always present in those with strong foot odour, but was never detected in those subjects without foot odour.

The reproduced foot odour was similar to that found *in vivo* and, as expected, was found to contain iso-valeric acid together with other short-chain fatty acids. It is significant that a pungent foot-like odour was generated upon addition of hydrochloric acid to odourless samples. We can speculate that short-chain fatty acids were in the form of their odourless metallic salts, which dissociated into free fatty acids on acidification. This may serve as a clue to why some individuals have strong foot odour whereas others do not. The mechanism by which subjects with weak or no body odour possess short-chain fatty acids in the metallic salt form is unknown. It may be possible to apply this finding to a deodorizing mechanism, and to convert offensive short-chain fatty acids into odourless metallic salts.

In this study we have analysed foot odour in respect of short-chain fatty acids that have also been identified in other body sites, such as the axilla,[4] vagina,[5] and hair and scalp.[6] Although body odours are mixtures of many chemical compounds, short-chain fatty acids contribute a large amount to foot malodour.

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Telogen skin contains an inhibitor of hair growth

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SUMMARY

We have investigated whether C 57 B1–6 mouse skin with all its follicles in the telogen stage of the hair cycle contains a hair-growth inhibitory activity, as opposed to skin with anagen follicles. Crude aqueous extracts of whole telogen mouse skin (TE), anagen skin (AE) or vehicle alone (V) were injected intraperitoneally into mice in which anagen had previously been induced by plucking of telogen hair follicles. Injection of TE, but not AE or V, significantly retarded the development of anagen follicles, as measured by macroscopic and quantitative microscopic hair growth parameters (skin pigmentation and thickness, appearance of trichohyaline granules) and the incorporation of tritiated thymidine into mouse skin from animals previously treated with either TE or V (skin organ culture). This inhibitory activity seemed to be localized to the epidermis and was also present in rat epidermis. We suggest that this apparently non-species-specific inhibitor present in telogen skin may play a role in regulating the hair cycle in rodents.

The development and cyclic activity (telogen, anagen, catagen) of the hair follicle require tightly controlled, mesenchyme-dependent epithelial cell proliferation.[1–3] Experimental systems which focus on hair growth provide models for the study of growth control and epithelial–mesenchymal interactions, in addition to their relevance for hair disorders. We have developed simple *in vivo* and *ex vivo* assays for the study of hair growth inhibition using C 57 B1–6 mice, which complement a previously suggested *in vivo* assay for hair growth stimulation,[4] and have investigated whether endogenous inhibitors play a role in regulating the hair cycle.

With induction of anagen, the skin pigmentation of C 57 mice progresses from pink (telogen) to black (late anagen), because the truncal skin melanocytes in these animals are confined to the hair follicles. Active melanogenesis is coupled with follicular growth[5,6] and the stage of the hair cycle can be assessed on the basis of skin colour alone. The development of anagen can be followed by recording changes in skin colour and skin thickness (the whole skin thickens in these mice as anagen progresses[5,7]) and the appearance of trichohyaline granules, an anagen-specific differentiation product of the follicle inner root sheath.[8] The follicles of these mice grow synchronously and can be induced to grow by stripping of the telogen hair shafts with a wax–rosin mixture, as plucking of telogen follicles leads to a traumatic induction of anagen.[9,10] This

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provides a simple system for studying the inhibition of rapidly proliferating epithelial tissues in general, and hair growth in particular.

Previous work from our laboratory suggested an inhibitory control mechanism for hair growth.[4] We used this model to study the presence of a local, endogenous hair-growth inhibitor in telogen mouse skin. It was proposed by Chase[6] that the cyclic activity of the hair follicle could be explained by the gradual build-up of inhibitor of proliferation during anagen. Eventually, the build-up of inhibitor would terminate anagen and lead to telogen, while the stimulus for the initiation of follicular activity could lie in the gradual loss of that inhibitory activity during telogen. According to this theory, telogen skin but not anagen skin should be rich in this hair-growth inhibitory activity. From our studies we have found that telogen skin contains an inhibitor of hair growth which supports the hypothesis of the role for endogenous agents in the control of the hair cycle.

METHODS

Animals

C 57 Bl–6 mice (female, syngenic, 6–8 weeks old, 15–20 g, and the telogen state judged by their pink skin colour), and long Evans hooded rats (female, 2–4 months old) were purchased from Charles River, Kingston, N.Y. For anagen induction the telogen mice were anaesthetized (30 mg sodium pentobarbitol/kg body weight) and stripped of hair using a wax and rosin mixture which was painted over the back of the mouse and after hardening, peeled off, inducing the resting follicles to enter anagen. Animals were then killed using ethyl ether.

Extract preparation

For the preparation of telogen (TE) or anagen (AE) skin extract, individual mice (telogen or anagen-induced mice, stripped 3 days before) were killed and then clipped. They were then washed briefly in 80% ethanol and the skin used for extract preparation (TE: whole torso; AE: anagen-induced back skin) was dissected free at the level of the subcutis. After two washes in PBS at 4°C, the skin of one mouse torso was immediately transferred to a 50-ml glass beaker containing 25 ml (AE: 20 ml) of 10 mM HCl (4°C), minced with scissors and homogenized. This homogenate was transferred to 15-ml polypropylene centrifugation tubes (Corning, N.Y.) and incubated for 1 h at 4°C. The crude extracts were then centrifuged in a table top centrifuge at 1500 rpm for 20 min at 4°C. The supernatant was removed and transferred into clean polystyrene tubes and stored at 4°C and used on the same day. Protein determination[11] of three representative extract preparations gave a mean concentration of 6·34 ± 0·64 (SDM) mg protein/ml for TE, and 5·42 ± 0·71 mg protein/ml for AE, using bovine serum albumin (Sigma Chemicals) as standard.

Extracts from telogen epidermis (TEE) and telogen dermis (TED) were prepared as above from whole telogen skin (mouse, rat) that had been cut with a scalpel into 5-mm wide strips and incubated overnight at 4°C in 10 mg/ml dispase (Boehringer Mannheim, F.R.G.) so that epidermis and dermis could be easily separated. The separated epidermis and dermis were then washed twice in PBS. One batch of TE was also dialysed overnight at 4°C in cellulose dialysis tubing (mw cut off: 12,000–14,000) against 4 l of 10 mM HCl and the dialysand used for injections (dialTE).

Mouse in vivo anagen induction assay

Anagen-induced mice were injected intraperitoneally (i.p.) once daily at 8–10 a.m. on days 0–5 with 0·5 ml of TE, AE, TEE, TED, ratTEE, dialTE or vehicle alone (V; 10 mM HCl), the first

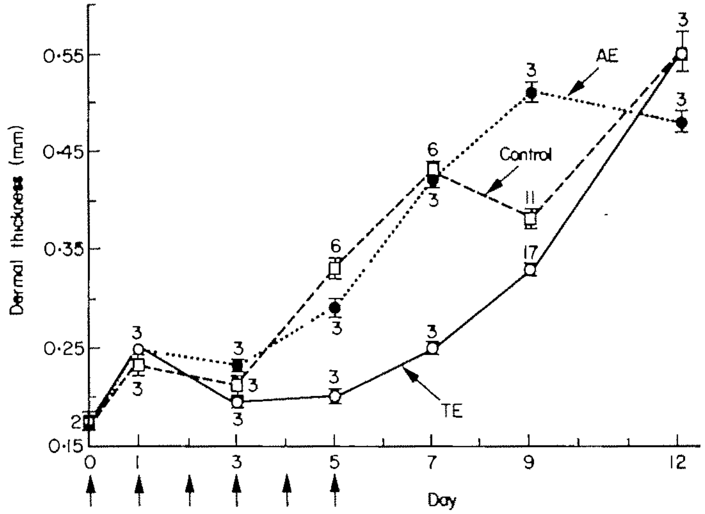

FIGURE 1. Measurements of dermal thickness (basement membrane to upper margin of panniculus carnosus) as an indirect parameter of anagen development after once daily intraperitoneal injection of telogen extract (TE, ○), anagen extract (AE, ●), or vehicle alone (control, □) into anagen-induced C 57 Bl-6 mice (the arrow sign represents i.p. injection of the respective extract, small numbers above each point = *n*; bars denote ± 1 SEM)

injection being within 3–5 h of anagen induction. Randomly selected animals were killed after 1, 3, 5, 7, 9 or 12 days at 5–7 p.m. for histology and their skin pigmentation and palpable skin thickness were recorded.

Two experimental protocols were used. In one set of experiments (dynamic assay) the effect of once daily i.p. TE injection (days 0–5) on anagen induction was followed over time and compared with the effects of AE and V (controls) (see Fig. 1). In another assay (static assay), different extract preparations (TE, AE, TEE, ratTEE, TED, dialTE, V) were injected into anagen-induced mice once daily (days 0–5); all animals were killed on the evening of day 5 and the dermal thickness was measured microscopically as an indirect quantitative parameter of anagen.

Histology

Mouse skin was harvested and processed for light microscopy, and dermal thickness was measured semi-blind as the distance between the epidermal basement membrane and upper margin of the panniculus carnosus, as previously described.[4] The presence of anagen-specific trichohyaline granules (THG) was measured semi-blind by assessing six microscopic fields (100 × magnification) per section, four sections per animal (expressed as the number of fields positive for THG per six fields (± SEM)). The thickness of untreated telogen skin was determined as 168 μm ± 3 SEM, while anagen skin 12 days after anagen induction by stripping gave a value of 550 μg ± 10 SEM.

Ex vivo assay: mouse anagen skin in organ culture

After twice daily i.p. injections of TE or vehicle on days 0–3 or on days 0–5 into anagen-induced mice, the animals were killed, and 4-mm punch biopsies taken from the skin of their backs.

These biopsies of almost identical diameter and tissue volume were cultured for 24 h at the air–liquid interface (37°C, 5% CO_2, air, 100% H_2O) on filter paper and stainless steel grids, with nine in each culture dish and with 5 ml DME with 10% fetal bovine serum and with antibiotics and antimycotics in the culture medium. At 0 h all dishes were pulsed with 5 µCi tritiated thymidine/ml (^{3}H-Tdr) specific activity: 171 mCi/mg, over 24 h. At the end of the incubation, each skin fragment was washed in PBS, the epidermis and dermis separated by sodium bromide incubation (nine biopsies in 40 ml of 2 M NaBr for 120 min at 37°C), washed again in PBS, individually solubilized in 300 µl Soluene (Packard Instr.) and processed for liquid scintillation spectrometry, using Ultima Gold (Packard Instr.) as scintillant. As shown by previous autoradiography experiments,[12] Tdr incorporation into cultured skin fragments occurred almost exclusively into epidermal and epithelial bulb keratinocytes. Thus, radioactivity measurements in the dermal fragment represented primarily epithelial bulb growth. This agrees with the reported proliferation kinetics of the different skin cell populations.[13,14]

RESULTS

In vivo assays (i.p. injections of extracts)

In contrast to vehicle or AE, intraperitoneally injected TE retarded the development of mouse anagen follicles *in vivo*. As shown in Figure 1 (dynamic assay), the differences in anagen development between the three extracts (TE, AE, V), measured by assessing the anagen-related increase in dermal thickness, were not apparent until day 5 after a total of six i.p. injections of extracts. On day 5, TE-injected mice showed a dermal thickness of 200 (± 8 SEM) µm, while AE-injected mice reached 290 (± 10) µm, and V-injected mice (controls) 330(± 10) (P value for TE/V $<$ 0·001, P for TE/AE $<$ 0·005). On day 7, 2 days after injections had been stopped, the measurements exhibited maximal differences between the three groups: 250 µm (TE), 420 µm (AE) and 430 µm (V). Whether the difference in dermal thickness between the vehicle and AE-injected groups on day 9 (Fig. 1) is merely an expression of the degree of variability of anagen development in this bioassay or reveals specific biological properties of AE, awaits further clarification. By day 12, the skin of all groups had again reached comparable stages of anagen development.

The statistically significant differences in microscopically measured dermal thickness between the three groups on days 5 and 7 were also visible macroscopically: the back skin of TE-injected mice was palpably thinner and visibly less pigmented than of AE- or V-injected mice

TABLE 1. The effect of telogen extract on different hair-growth parameters at day 5. After once daily i.p. injection of either TE or V on days 0–5, all mice were killed, identical regions of back skin were processed for histology, and the dermal thickness as well as the presence of trichohyaline granules (THG; expressed as microscopic fields positive for THG per six fields) were measured

		Microscopic		Macroscopic	
n	6 × 0·5 ml i.p.	Skin thickness (µm ± SE)	THG (fields pos/6 fields)	Pigmentation	Thickness
4	Vehicle	250 ± 5	5·3 ± 0·6	Control	Control
5	TE	190 ± 4	1·1 ± 0·4	Decreased	Decreased

(Table 1). Interestingly, daily i.p. injections of TE over 12 days failed to completely block anagen development. Both TE- and V-injected mice exhibited a skin thickness of 510 μm in this experiment (SEM $< 1{\cdot}2$ μm, $n = 3$ per group) and showed the presence of THG in almost all microscopic fields examined.

In a separate experiment (Table 1: static assay), the i.p. injection of TE on days 0–5 after anagen induction was compared with the injection of vehicle alone (all animals killed on day 5). This assay confirmed the findings of the previous dynamic assay: TE-injection significantly inhibited the development of anagen follicles as compared to the controls. In particular, the appearance of THG, an additional highly specific marker for anagen follicles, was retarded by TE injection. In these mice less than 20% of the microscopic fields scanned exhibited THG in the follicle internal root sheath, compared to about 90% THG-positive fields in the control animals.

A second static assay compared the effects of i.p. injections of different extract preparation on days 0–5 on the dermal thickness of anagen-induced mice, all of which were killed on day 5. Table 2 shows that mice injected with V and TED exhibited significantly thicker skin than those mice injected with TE, TEE, ratTEE or dialTE, with the strongest inhibitory activity exerted by TE and dialTE.

Ex-vivo assay: exposed in-vivo/assayed in-vitro

The data from the *ex vivo* assay suggest that the *in vivo* effect of TE-injection (twice daily, days 0–5) persists and can be measured *in vitro* when, after finishing the injections and killing the animals on day 5, their skin is incubated for 24 h in an *ex vivo* skin organ culture assay.

Measuring the incorporation of ^{3}H-Tdr as a parameter for cell proliferation, the epidermis of V-injected mice gave 17955 (± 1146 SEM) cpm and the dermis 22156 (± 1385) cpm, where almost exclusively epithelial bulb cells proliferate during this incubation period, thus reflecting follicle growth.[4,12] The epidermis of TE-injected mice, in contrast, showed significantly more Tdr incorporation (36114 ± 6723) and the dermis significantly less Tdr incorporation

TABLE 2. The effect of different skin extracts on dermal thickness at day 5 Anagen-induced mice were injected i.p once daily on days 0–5 with different extract preparations. Control, 10 mM HCl; TE, whole telogen skin extract; TEE, telogen epidermis extract, TED, telogen dermis extract; dial TE, dialysand of dialysed TE

	Day post-stripping	Skin thickness (μm ± SE)	*n*
Untreated			
Telogen skin	0	168 ± 3	6
Anagen skin	12	550 ± 10	3
Extract-treated			
Control	5	293 ± 5	11
TE	5	180 ± 4	5
TEE	5	220 ± 5	4
TED	5	290 ± 7	5
Rat TEE	5	210 ± 3	5
Dial TE	5	190 ± 3	6

(11054 ± 1566) than the controls. However, when this *ex vivo* assay was performed with skin taken from animals that had been injected fewer times and were killed earlier (injections: twice daily on days 0–3, killed on day 3) no significant differences in ^{3}H-Tdr incorporation occurred between the skin of TE and V injected animals ($n = 9$ in all assay groups).

DISCUSSION

Using a simple *in vivo* assay for the study of hair-growth inhibition, we have shown that mouse telogen, but not anagen, skin contains an inhibitor that, on intraperitoneal injection into anagen-induced mice, retards the development of anagen follicles. This inhibitor appears to be derived from the epidermis, but is not species-specific, as an extract preparation from rat epidermis also slows anagen development in mice (Table 2).

The data from the *ex vivo* assay suggest that the telogen-specific inhibitor may be hair-growth specific, as it may actually stimulate epidermal proliferation rather than inhibit it. The *ex vivo* experiments also show that the effect of TE on anagen development and thus on epithelial bulb cell proliferation, persists for some time in the absence of systemic influences after the skin has been removed from the animal and grown in organ culture (^{3}H-Tdr pulse and incubation time: 24 h).

Only telogen, and not anagen, skin extracts retard the anagen development in the dynamic assay (Fig. 1), so our observations tend to support the concept that an inhibitory/disinhibitory mechanism plays a role in regulating the hair cycle.[3,4,6,12] It is not known whether the inhibitory activity associated with telogen skin can act directly on isolated follicles such as rat vibrissae follicles *in vitro*,[15] or requires the presence of systemic influences. Evidence as to whether it acts by delaying the onset of anagen or by retarding its development requires the development of more specific, sensitive and reliable assays for detecting the earliest molecular events of anagen induction in telogen follicles. Although the inhibitor contained in the crude aqueous skin extract needs to be characterized biochemically, it appears that it is a telogen-specific molecule. It may be related to the putative 'epidermal chalone'[16–17] in that it localizes to the epidermis and is a non-species-specific inhibitor. We previously reported that an epidermal-derived inhibitor of mouse epidermal cell proliferation, the pentapeptide pyroGlu-Glu-Asp-Ser-GlyOH,[18] non-species-specifically inhibits hair growth *in vivo* and *in vitro*.[12]

Our findings contrast with those of Bullough and Laurence[19] who did not observe an antimitotic action of aqueous mouse epidermal extract on hair bulbs. This discrepancy remains to be explained, although the different methods of extract preparation and the different animals used by these authors, as well as the potential lability of the inhibitor, may account for this. Frater[20] has reported the extraction of a lectin-like inhibitory activity from rat skin in an undefined stage of the hair cycle with a suggested MW of 5000–10,000. It remains to be established whether that inhibitory activity or the epidermal pentapeptide are related to the inhibitor as described by us.

We have controlled for the possibility that the hair-growth inhibitory activity of TE may simply be due to non-specific toxicity of our extract preparation by comparing the effects of an equal amount of anagen and telogen skin extract of similar protein concentration, in addition to administering a vehicle control. This study shows that there are indeed distinct differences in activity between AE and TE which are unlikely to be based solely on differences in the toxicity of these two extract preparations.

The development of anagen, once induced, could not be blocked completely by continuous injection of TE, though it could be considerably retarded in its earlier stages. The hair cycle program which 'switches on' anagen in resting follicles may be fairly resistant to the interference of endogenous mitotic inhibitors and may follow an all-or-none pattern of growth. Once initiated, the induction program appears to progress until development of the full anagen follicle is completed, an observation which is in accordance with the studies of Johnson and Ebling.[9] Alternatively, the temporary effect of the inhibitory activity contained in telogen skin might be explained by the increasing appearance of counteracting stimulatory factors during the course of anagen development, which may eventually override the inhibitory agent.

In summary, we have used simple and versatile assays for the study of hair-growth inhibition to demonstrate that mouse telogen skin contains a non-species-specific inhibitor of hair growth, which may play a role in the regulation of the rodent hair cycle. A purified inhibitor, as well as subsequently synthesized specific antagonists, should be clinically useful molecules, since they might serve as effective and specific therapeutic modulators for treating disorders of epidermal and follicular growth such as epidermal hyperplasia or skin atrophy and alopecia or hypertrichosis.

ACKNOWLEDGMENTS

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In vivo study of scleroderma by non-invasive techniques

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SUMMARY

Sixteen patients with localized scleroderma (LS) and 11 with progressive systemic sclerosis (PSS) were studied using non-invasive techniques to determine skin thickness, skin extensibility, transcutaneous PO_2 and cutaneous blood flow and were compared with normal controls. LS was characterized by a decreased skin extensibility with thickening of the skin in progressive lesions. There was an increase in the cutaneous microcirculation with a decrease in the PO_2. In PSS, there was a decrease in the skin extensibility caused by thickening of the skin, but no change in cutaneous microcirculation or PO_2 values. These results indicate that skin thickness and extensibility are the most useful parameters in the study of the progression of LS and PSS.

Localized scleroderma or morphoea is characterized by well-defined plaques and bands or drop-like lesions that are easily recognized. The classical appearance is that of a central atrophic area which is either pigmented or of a pearl-white colour and with a lilac-coloured border. These plaques may blend with normal skin but usually remain detectable on palpation. In PSS, large areas of the skin appear to be thickened although this can be difficult to evaluate in the early and intermediate stages. The quantitative evaluation of scleroderma by the use of objective parameters is of interest as regards diagnosis and monitoring progression, and also for assessing the effectiveness of various therapeutic agents.

Previous studies using non-invasive techniques have been carried out in scleroderma as regards skin elasticity,[1] skin thickness,[2] skin colour[3] and studies on the microcirculation.[4] These have been mainly on the localized form of scleroderma and have shown that the plaques are more thick and less extensible than the normal skin and that the microcirculation is greater in the centre of the lesions. In this study we have applied several techniques to the same lesions with the aim of identifying the most sensitive parameter for the changes in the skin in scleroderma.

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METHODS

Lesions studied

Localized scleroderma (LS). Seventeen lesions were studied in 16 patients (mean age: 33·5 years; range: 7–72 years). The clinical details are given in Table 1. Five of the lesions were considered progressive with the presence of a lilac border and/or an increase in size and/or the appearance of new lesions. Five lesions were resolving either by a regression or disappearance of the lilac border or a reduction in pigmentation between the examinations. Seven lesions were considered to be static. Sixteen months after the end of the study the lesions were re-examined and the central parts were measured and compared with contralateral non-involved areas.

Progressive systemic sclerosis (PSS). Eleven cases of systemic scleroderma were studied. They were classified according to Winkelmann,[5] and five were type I which were weakly progressive, four were type II which were highly progressive. One case was associated with dermatomyositis and had the mixed connective tissue disorder, and one patient with progressive systemic scleroderma progressed to develop eosinophilic fasciitis (Shulman's syndrome). The clinical details of these cases are given in Table 2. The site investigated was the ventral surface of the forearm. The results were compared to those obtained under the same conditions from a group of healthy controls (approximately 10 subjects in each 10-year age range).

TABLE 1. Clinical details of patients with localized scleroderma

	Single morphoea	Multiple morphoea	Single band	Multiple bands
Number of patients	8	4	1	4
Lesions clinically				
progressive	1	3	0	1
regressive	7	1	1	3

TABLE 2. Clinical details of patients with progressive systemic sclerosis

No.	Patient	Age (years)	Sex	Type of scleroderma	Duration of disease (years)	Organ involvement					
						Raynauds	Cardiac	Lung	Gut	Arthri.	Renal
1	C.B.	44	F	Type I	13	+	0	0	0	+	0
2	A.B.	47	F	Type I	8	+	0	0	0	0	0
3	C.C.	69	F	Type I	24	+	0	0	0	0	0
4	N.C.	38	F	Type I	2	+	0	0	+	+	0
5	M.G.	41	M	MCTD	2	0	0	0	0	+	0
6	C.G.	62	F	Type II	10	+	+	+	+	+	+
7	G.M.	30	F	Type II	15	+	0	+	+	0	0
8	M.P.	77	M	Type I	9	+	0	0	+	0	0
9	M.P.	52	F	Type II	15	+	+	+	+	0	0
10	L.R.	57	F	Eos. Fac.	6	0	+	0	+	0	0
11	Y.S.	22	F	Type II	2	+	0	±	+	+	0

Measurement of skin extensibility

This parameter was assessed by a torque method using a twistometer®.[6,7] Briefly, a low torque (30×10^{-4} Nm) was applied with a disc with a diameter of 18 mm that was fixed to the skin using double-sided adhesive tape. A guard ring limited the width of the ring of skin studied to 3 mm. The angle of rotation was measured by a detector (RVDT) and displayed automatically. In the experimental conditions used the extensibility of the entire skin, both dermis and epidermis, was measured.[7]

Skin thickness measurement

The skin thickness was evaluated using the ultrasound A-scan echography technique.[8] Briefly, an ultrasound pulse was applied at the skin surface and was reflected by the dermis–hypodermis interface. The transit time was measured and displayed by means of an oscilloscope. The skin thickness was derived from the speed of ultrasound in skin, i.e. 1605 m/s.[9]

Cutaneous microcirculation

The cutaneous microcirculation was measured using a laser Doppler velocimeter (LDV Periflux). This technique specifically measures blood flow. Each value was the mean of three determinations taken at 5 intervals.

Transcutaneous PO_2 measurements

A Roche Kontron apparatus was used to measure the PO_2 in a carefully cleaned area with the probe at 44°C. The probe was calibrated in the room taking into account the atmospheric pressure and was zeroed in a current of nitrogen gas. The results were expressed in mmHg.

Statistics

The results were analysed using a *t*-test for paired values in LS and a *t*-test for two mean values in PSS. The values were expressed as mean ± SEM.

RESULTS

Localized scleroderma

The skin thickness and extensibility of progressive and regressive lesions are shown in Figures 1 and 2, respectively. In the case of progressive lesions, the mean skin thickness was 60% ± 5% greater than in contralateral areas ($P < 0\cdot04$), whereas extensibility was reduced by 67% ± 11% ($P < 0\cdot001$). For regressive lesions the values for thickness were − 10% ± 6% and for extensibility were − 35% ± 10% ($P < 0\cdot005$).

The results for cutaneous blood flow and PO_2 are given in Figures 3 and 4. Blood flow was increased by approximately 40% ± 10% in the centre of both progressive and regressive plaques ($P < 0\cdot001$). PO_2 was decreased in progressive lesions by 35% ± 10% ($P < 0\cdot01$) and by 17% ± 5% ($P < 0\cdot01$) in the regressive lesions.

Generalized scleroderma

The results for skin thickness and extensibility relative to controls are given in Figure 5. The skin thickness was approximately twice (+ 106% ± 18%) that found in age and sex-matched controls and extensibility of the skin was reduced by 68% ± 8%. Both of these differences were highly significant ($P < 0\cdot001$). No significant differences in the cutaneous blood flow or PO_2 were observed relative to the controls.

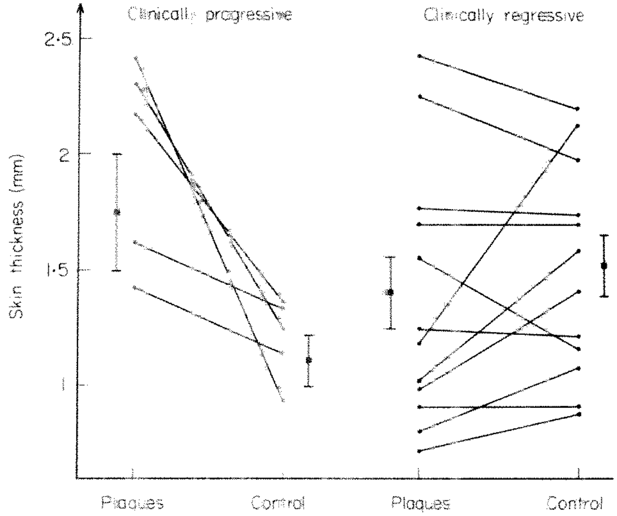

FIGURE 1. Skin thickness in the centre of the plaques, compared to controls, for progressive and regressive lesions of LS.

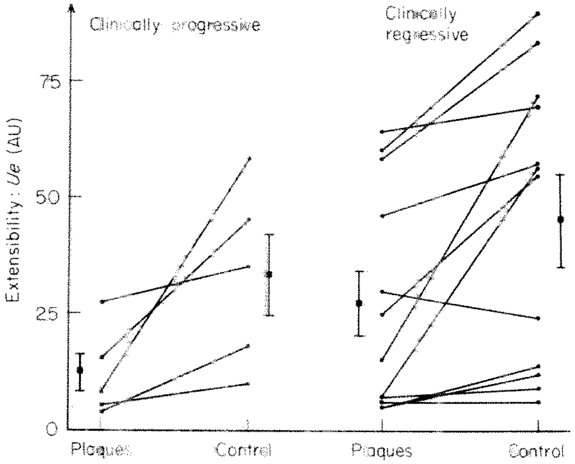

FIGURE 2. Skin extensibility assessed in the centre of the plaques, compared to controls, for progressive and regressive lesions of LS.

DISCUSSION

Previous attempts to characterize sclerotic skin using objective criteria have mainly involved measurements of its visco-elastic properties. A study using a relatively simple device for the measurement of skin extensibility in the vertical plane, showed that it was reduced by up to 75% in acrosclerosis.[1] Using a similar device which enabled repetitive stress to be applied perpendicularly to a disc of skin, it was shown that the distensibility of sclerodermic plaques in LS was strongly reduced, as were hysteresis and resilient distension, although resilience was increased in regressive plaques.[2] It was also shown, using A-scan ultrasound, that skin thickness

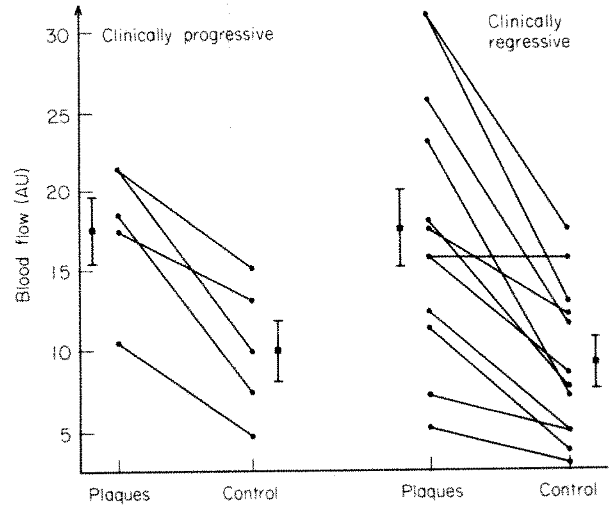

FIGURE 3. Cutaneous blood flow measured in the centre of the plaques, compared to contralateral areas, for progressive and regressive lesions of LS.

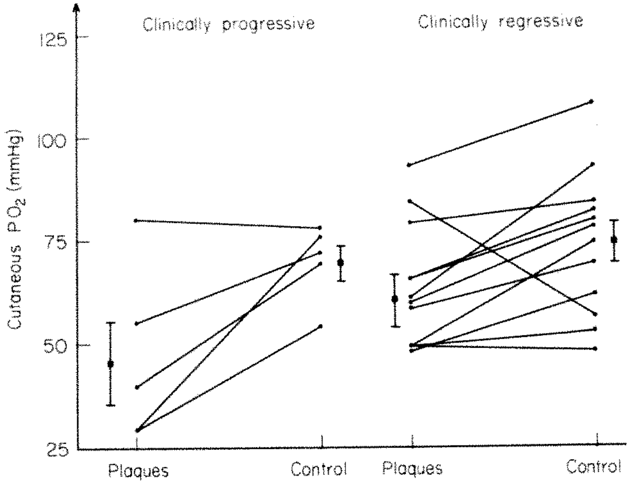

FIGURE 4. Transcutaneous PO_2 measured in the centre of the plaques, compared to contralateral areas, for progressive and regressive lesions of LS.

in the centre of the plaques was clearly increased relative to contralateral areas, particularly in the case of advanced scleroderma.[2]

The results in general were confirmed in our study. However, the clinical differentiation of LS plaques in terms of their extensibility allowed a more precise characterization of the affected skin. The extensibility of skin, as with other materials, depends mainly on its thickness, and under strain depends on the following parameters:[10] $E = M/2\pi\ 0{\cdot}4\ R1\ R2 \times 1/(Ue \times Ep)$, where E is the elasticity modulus of the tissue, Ep is thickness, $R1$ and $R2$ are the geometric

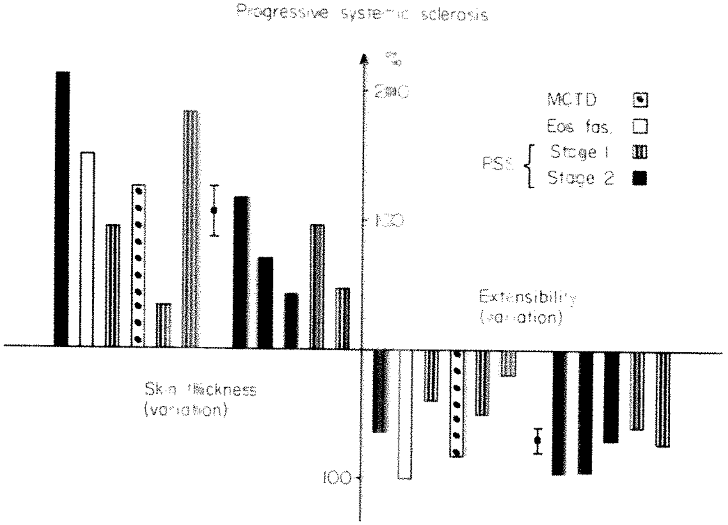

FIGURE 5. Variation (%) in skin thickness and skin extensibility compared to control groups for cases of PSS according to their clinical classification.

characteristics of the device and Ue is the skin extensibility. The same formula gives the following relation by differentiation: $\Delta E/E = -\Delta Ue/Ue + \Delta Ep/Ep$. In the case of progressive lesions, this leads to a negligible variation in the modulus: $\Delta E/E = (-0{\cdot}58 + 0{\cdot}52) = -0{\cdot}06$ (NS), while in the case of regressive lesions: $\Delta E/E = -(0{\cdot}39 - 0{\cdot}10) = +0{\cdot}49$ ($P < 0{\cdot}001$). This latter increase in the modulus reflects an intrinsic increase in skin stiffness, whereas in the former case the reduced elasticity would appear to be due solely to an increase in skin thickness. The same approach can be used to study the characteristics of PSS. In this case, considering the lesser accuracy of the measurements because of the use of a control group, the reduction in skin elasticity can be explained by the large increase in skin thickness.

With regard to blood flow measurements, there is little published data, but Serup[3] and Kristensen and Wadskov[11] have shown that in LS cutaneous blood flow was increased in the centre of the plaques. This increase correlated with clinical severity and was confirmed by our studies, although an increase was also observed in regressive lesions. However, it should be noted that regressive lesions remain sclerotic, as shown by the measurements of skin elasticity. Partial pressure of oxygen, on the contrary, was reduced. This reduction was more marked in progressive (34%) than in regressive plaques (18%). Laser Doppler blood flow (LDV) measurements reflect overall flow in the deep arteriolar network, with the exception of the capillary vessels themselves, where flow is extremely weak. Partial pressure of oxygen was evaluated at the skin surface and corresponded to the oxygen supply through the arterial walls minus oxygen consumption in the dermis and epidermis. Oxygen diffusion through a fibrotic dermal network may well be restricted, while the reduction in partial oxygen pressure measured at 44°C, associated with an increase in blood flow, would reflect increased oxygen consumption by the tissues. This phenomenon is characteristic for inflammation.[12,13] The smaller reduction in PO_2 in regressive lesions would reflect the lower degree of inflammation. The divergence between these two parameters (LDV and PO_2) could also be explained by a reduced vascularity of the papillary dermis.[14] This would be compensated by a higher blood flow in the dermal plexus. Unfortunately, there are no specific methods for studying the microcirculatory network.

Finally, this study has shown that the largest and most significant differences between sclerotic and control skin were obtained by means of measurements of skin thickness and elasticity. The two techniques, A-scan ultrasound and twistometer® were of considerable use in the study of the pathology and progression of scleroderma and can be used to evaluate the improvement due to effective therapeutic agents.

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Increased stratum corneum turnover induced by subclinical irritant dermatitis

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SUMMARY

The chronic effects of the irritant sodium lauryl sulphate (SLS) on stratum corneum (SC) barrier function, determined by transepidermal water loss (TEWL) measurements and on epidermal cell kinetics, estimated by stratum corneum turnover time (SCTT) determination (dansyl chloride staining method), were investigated in 18 healthy female volunteers. SLS (7·5%) was applied without occlusion for 20 min once daily, over a period of 3 weeks (5 days a week) on dansyl chloride-stained skin and on untreated skin. SCTT of untreated skin (19·3 ± 0·8 days; mean ± SEM) was not changed by daily treatment with water (control) (19·3 ± 2·0) but was significantly reduced by SLS (10·9 ± 0·6; $P \leq 0{\cdot}0001$; compared to controls). However, TEWL was increased in SLS-treated sites 1·5-fold after 4 days of treatment (5·3 ± 0·6 vs. 3·5 ± 0·3; $P < 0{\cdot}001$). At the end of the second week, TEWL was increased 2·6-fold and after 3 weeks TEWL was 3·3 times higher than in controls (13·0 ± 1·6 vs. 3·9, $P \leq 0{\cdot}0001$). The intensity of SLS-induced irritation as measured by TEWL was significantly correlated with baseline TEWL ($r = 0{\cdot}50$; $P \leq 0{\cdot}02$) and significantly negatively correlated with SCTT of SLS treated sites ($r = -50$; $P \leq 0{\cdot}02$) but not with SCTT of untreated skin ($r = 0{\cdot}19$).

Although irritant contact dermatitis can produce significant disability, relatively little is known about its pathophysiology.[1–3] Many studies concerned with cutaneous irritation use a single 24–48 h occlusive application of sodium lauryl sulphate (SLS) as a model. This is useful in studying the physiology of irritated skin, but surfactant exposure in real life is usually of shorter duration and cumulative. We therefore investigated the chronic effects of the model irritant, SLS, on stratum corneum (SC) barrier function, determined by transepidermal water loss (TEWL) measurements and on SC turnover time (SCTT), as estimated with the dansyl chloride

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fluorescence technique. SLS-induced TEWL increase was compared with baseline TEWL, SCTT of healthy skin and SCTT of SLS-treated skin.

METHODS

Study population

Eighteen healthy, Caucasian women aged between 18 and 40 years were the volunteer subjects after having given their written informed consent. The study had been approved by the University of California San Francisco Committee for Human Research.

SC turnover time (SCTT)

SCTT was determined using the dansyl chloride staining method.[4] Briefly, stratum corneum proteins were stained with fluorescent dansyl chloride (5% in petrolatum for 24 h). Subsequently, the treated skin was examined daily with ultraviolet illumination. SCTT was the time in days from staining until fluorescence disappearance.

Applications

SLS (7·5%) in distilled water was applied to premarked test sites on the mid volar forearm (2·5 cm^2) without occlusion for 20 min once daily (5 days a week for 3 weeks) on dansyl chloride stained skin and on untreated skin: 0·2 ml was pipetted into a glass ring (1·8 cm inner diameter) which was fastened to the skin with silicone (Adhesive Clear, Dow Corning Corp. Midland, MI). Water served as vehicle control.

Measurements

TEWL was measured before application of the solution at days 0, 2, 4, 9, 11, 16 and 18 with an Evaporimeter (Servo Med, Stockholm, Sweden). This instrument uses the method of vapour pressure gradient calculation.[5] The probe was held in place for each measurement until a stable TEWL value had been established (approximately 30 s). Before measurement, all volunteers had been resting at room temperature (20 ± 2°C; 45–65% relative humidity) for at least 15 min. Each site was also examined and graded according to a 5-point visual scoring system: 0, no erythema; 0·5, equivocal reaction; 1, slight redness, either spotty or diffuse; 2, moderate, uniform redness; 3, intense erythema; 4, fiery red with oedema.[6]

Statistics

Differences in TEWL and SCTT were tested for their significance with Student's *t*-test for paired samples.[7] Correlation coefficients were calculated according to Pearson.[7]

RESULTS

Daily application of 7·5% SLS for 20 min significantly increased TEWL after 4 days of treatment (5·3 ± 0·6 vs. 3·5 ± 0·3; means ± SEM; $P \leq 0{\cdot}001$). At the end of the second week, TEWL was increased 2·6-fold; after one more week TEWL was 3·3 times higher than in controls (Fig. 1). Only two of 18 volunteers showed an equivocal reaction at the end of the first week (Table 1); after one more week, half the volunteers showed some erythematous reaction. At the end of the study two-thirds of the study population showed erythema with a mean erythema score of 1·0 (Table 1).

SCTT of untreated skin (19·3 ± 0·8 days) was not altered by daily treatment with water (19·3 ± 2·0) but was significantly reduced to 10·9 ± 0·6 by chronic SLS application (Fig. 2).

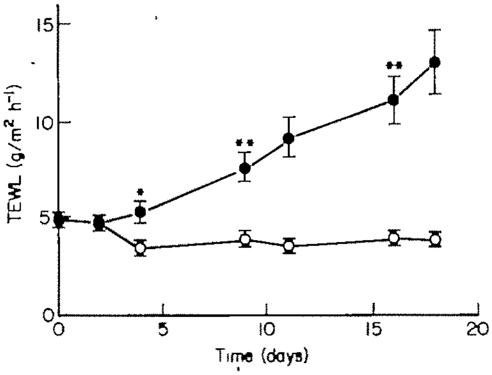

FIGURE 1. TEWL g/m^2 h^{-1} was measured before daily application (5 days a week) of 0·2 ml 7 5% SLS (●) or water as vehicle control (○) to volar forearm skin. Note that TEWL was significantly increased in SLS-treated sites as compared to controls prior to onset of erythema in most subjects (day 4). *$P \leq 0\ 001$; **$P \leq 0\ 0001$ (paired *t*-test) (means ± SEM; *n* = 18 volunteers)

TABLE 1. Erythema scores

Day	0	0 5	1	2	3	4	Mean ± SEM
0	18	0	0	0	0	0	0 0±0 0
2	18	0	0	0	0	0	0 0±0 0
4	14	3	1	0	0	0	0 1±0 1
9	13	2	2	1	0	0	0 3±0 1
11	9	3	5	1	0	0	0 5±0 1
16	5	4	6	3	0	0	0 8±0 2
18	5	1	6	6	0	0	1 0±0·2

Erythema scores (arbitrary units) before daily application (20 min) of 7 5% SLS to volar forearm skin (*n* = 18 volunteers).

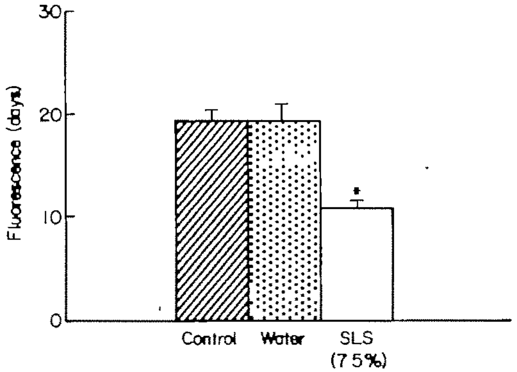

FIGURE 2. Stratum corneum turnover time (SCTT) estimated with the dansyl chloride staining method. SCTT of untreated skin (19·3 ± 0·8 days; means ± SEM) was not altered by daily treatment with water (control) (19·3 ± 2 0) but was significantly reduced by daily SLS application *$P \leq 0{\cdot}001$ (paired *t*-test; *n* = 18 volunteers).

TABLE 2. Correlation matrix

	TEWL 1	TEWL 2	SCTT 1	SCTT 2
TEWL 1	1			
TEWL 2	0·501*	1		
SCTT 1	0·040	0·193	1	
SCTT 2	0·014	0·500*	0·519*	1

Correlation coefficients between pretreatment TEWL (TEWL 1; mean of two replicates), TEWL after 3 weeks of cumulative SLS exposure (TEWL 2), SCTT of untreated skin (SCTT 1) and SCTT of SLS-treated skin (SCTT 2) ($n = 18$ volunteers).

* Significant at $P \leq 0{\cdot}02$.

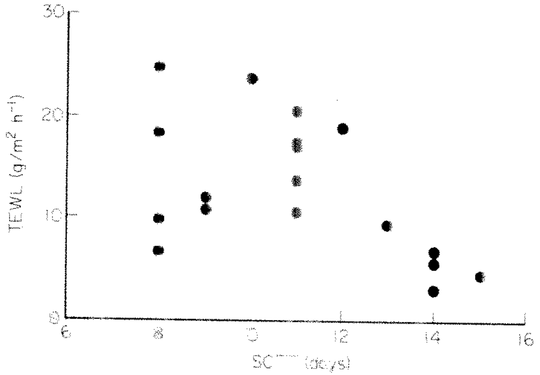

FIGURE 3. TEWL of SLS-treated sites (after 3 weeks of daily open exposure to 7·5% SLS for 20 min) was significantly negatively correlated with stratum corneum turnover time (SCTT) of SLS-exposed skin ($n = 18$ volunteers). $r = -0{\cdot}50$; $P \leq 0{\cdot}02$.

No significant correlation between SCTT of untreated skin and SLS-induced TEWL increase was seen (Table 2). However, baseline TEWL and SCTT of SLS-treated skin were significantly correlated with TEWL of SLS-irritated skin (Table 2, Fig. 3).

DISCUSSION

There have been several studies of the effects of an acute single application of diverse irritants on skin physiology and epidermal kinetics.[8-11] However, exposure to irritants, especially surfactants, occurs differently in everyday life. Exposure tends to be cumulative over weeks to years, with only several minutes each day and usually without occlusion. It is not known whether the pathophysiology of irritant dermatitis induced by a single 24-h patch application is comparable with chronic irritant dermatitis. One study demonstrated decreased SC water binding capacity induced by open SLS application for three consecutive days,[12] whereas a 24-h patch exposure to SLS was reported to increase the SC water content and water binding capacity.[13] There may be more differences between acute and chronic irritant dermatitis. The Soap Chamber Test[6] and the 21-day human-cumulative-irritation-assay[14] both employ repeated exposure of the same skin site to an irritant, but both tests use occlusive application to amplify the effect. Their purpose is to detect and quantify mild to moderate irritant consumer products rather than to study the subclinical changes induced by irritants.

In this study we investigated the effects of cumulative SLS exposure on SC barrier and epidermal kinetics of human skin. Measurement of TEWL allowed detection of subclinical changes occurring in the absence of erythema as, in the majority of volunteers (day 4), TEWL was increased in SLS-treated sites before a clinical effect was noticeable. As treatment continued, TEWL increased further and erythema was seen in more of the volunteers.

The effects of this treatment on SCTT were also investigated. The dansyl chloride method[4] is an indirect method for estimating epidermal kinetics, and relies on subjective evaluation, but it is easy to perform, non-invasive and provides a reasonable estimate of SC renewal.[4,11,15-17] As the transit of cells through the horny layers is a function of the rate of cell production by dividing cells, SCTT is an indirect measure for mitotic activity.[4] Our SCTT data of untreated skin (19·3 ± 0·8 days) agrees with previous data (17·7–18·5 d).[4,15-17] Treatment with water for 20 min daily produced no change in SCTT, whereas SLS-treatment induced almost a 50% reduction in SCTT. Thus, skin kinetics, i.e. mitotic activity, was stimulated before irritation was visible in most volunteers. It is unlikely that the faster disappearance of the fluorescence dye was due to a washing effect unrelated to SLS-induced irritation, as twice daily washing with soap for 1 min did not change SCTT,[4] and the reduction in SCTT was significantly correlated to changes in the SC barrier induced by the same treatment. The cosmetic industry markets products claimed to 'rejuvenate' the skin, and uses the dansyl chloride method as evidence of their efficacy. We suspect that the increased cell turnover noted may be related as much to irritation as to 'rejuvenation' of the skin.

After 3 weeks' treatment with SLS, TEWL showed significant linear correlation with pretreatment TEWL values. This supports earlier studies[18,19] that baseline TEWL is predictive for the severity of surfactant-induced irritation. However, we were not able to find this correlation in earlier studies when a 24-h occlusive SLS application was employed.[20]

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Dermatological findings in children exposed transplacentally to heat-degraded polychlorinated biphenyls in Taiwan

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SUMMARY

We studied 128 children who were transplacentally exposed to polychlorinated biphenyls and dibenzofurans in Taiwan, their parents and siblings who were directly exposed, and 115 control children. Direct exposure of the mothers stopped in 1979 and the children were born as late as 1985. At birth, exposed children had increased rates of hyperpigmentation, eyelid swelling and discharge, deformed nails, acne, natal teeth and swollen gums compared to controls. On examination, they had a much higher rate of dystrophic finger-nails and pigmented or dystrophic toe-nails than controls. They also had an increased rate of hyperpigmentation and acne. In addition they had more generalized itching, localized skin infections and hair loss. The findings seen in transplacentally exposed children differ from those seen in people directly exposed, particularly in the latter group in higher prevalence of acne.

In the spring of 1979, there was an epidemic of acne with other symptoms among boarders at a school for blind children in central Taiwan. Within a few weeks, cases occurred in local factories and later on throughout Changhua and Taichung counties. A retrospective study indicated that the earliest cases were from December 1978. An epidemiological investigation together with analytical chemical studies indicated that the illness was a form of chloracne, due to the ingestion of rice-bran cooking oil that had been contaminated during manufacture with a mixture of polychlorinated biphenyls (PCBs).[1] The PCBs had been repeatedly heated and cooled and were contaminated by the highly toxic and acnegenic polychlorinated dibenzofurans (PCDFs).[2,3] Distribution of the contaminated brand of oil was stopped in October 1979. A registry of

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exposed persons maintained by the health department eventually included over 2000 people. This was the second recorded epidemic of chloracne due to PCB contamination of cooking oil; the first had occurred in Japan in 1968 and involved over 1000 cases.[4]

The dermatological findings in adults who ingested the oil in Japan[5] or Taiwan[6] and in persons with industrial chloracne[7] are well described, but there is very little information on the offspring of female patients.[8] Because the PCBs and PCDFs are stored in fat tissue and are metabolized and excreted very slowly, the offspring of female patients continued to be born affected, even though acute direct exposure ceased after the oil was recalled in the autumn of 1979. Taiwanese health department registry data up to February 1983 included 39 hyperpigmented children born to exposed mothers, of whom eight had died.[1] To characterize the transplacental syndrome clinically and epidemiologically, we performed a field survey in Taiwan in April 1985 of all living children of exposed mothers who were known to have been *in utero* during or after the period of oil contamination. These children would have had transplacental and possible exposure through breast milk, but would not themselves have consumed the contaminated oil. We have reported the general findings of this survey elsewhere,[9] but in this report describe the dermatological findings in greater detail.

METHODS

In January 1985, all living children were identified who had been born to mothers included in the registry, informed concent was obtained, the mother interviewed, and the family invited to attend a clinic in April. Any of the children born in or after June 1978 were considered to have been at risk of transplacental exposure. There were 132 such living children, born to 74 mothers and they ranged in age from a few months to just under 7 years old. The mothers were interviewed in January about their child's health, their own exposure to the toxic rice oil, and their symptoms during the pregnancy. During the April examinations, mothers (or occasionally fathers or grandmothers) filled out an additional history form specifically for the dermatological findings as experienced by themselves and by their children. The examinations were carried out over 11 days in April 1985 at four sites in north and central Taiwan. Almost all patients were seen by JST; about 25% were also seen by YCW, and the parents were usually present. The physical examinations were conducted without knowledge of medical history. However, as the families knew each other and some adults still had obvious chloracne, blinding was not possible.

Information was obtained from the interviews on 128 of the transplacentally exposed children (four infants were missed). Of these, 117 children from 68 families attended the clinic; one infant died between interview and examination. The dermatology checklist for one exposed child was not obtained. In addition, in each exposed family, the youngest sibling born before June 1978 (if any) was also invited. There were 28 of these older siblings; 16 were interviewed and 15 came to the clinic. Most of these siblings had consumed the contaminated oil, but none had transplacental exposure. Most of the exposed adults were also examined, but not usually over the whole body.

The family was asked to suggest possible control children of the same age and from the same neighbourhood. The same questionnaire and examinations were administered to these mothers and children. The mothers were asked about exposure to confirm that they were unexposed to the contaminated oil. Mothers of 115 control children from 96 families were interviewed and, of these, 108 children from 92 families attended the clinic. Two control children left the clinic before reaching the dermatology station. No controls were obtained for the older siblings or the adults.

Statistics presented are usually frequencies and percentages. Proportions were compared by

Fisher's exact test; the role of covariates such as the date of birth and the number of older siblings also transplacentally exposed was examined through logistic regression.

RESULTS

Neonatal history as reported by parents

The transplacentally exposed children had a number of findings as reported by their parents. They had a history of eyelid swelling and discharge, acne neonatorum, nail deformity, and hyperpigmentation at birth which was much more frequent than in older siblings or the unexposed children (Table 1). Only transplacentally exposed children were reported to have natal teeth or swollen gums at birth. Of the 57 hyperpigmented children, 46 (all transplacentally exposed) were reportedly pigmented over their entire body. The most common sites for acne in the transplacentally exposed were the forehead (eight children), trunk (eight), and cheeks (seven). One sibling had acne on the cheeks only. All of these (as shown in Table 1) were significantly more in the transplacentally exposed children than in controls (Fisher exact test, $P < 0\cdot001$).

Many children had multiple neonatal findings reported. There were 75 children with at least one of the findings as listed in Table 1; 13 (including one control) had only hyperpigmentation, seven (including five controls) had only eye discharge, three had only swollen gums, one had only acne, one had only deformed nails, and 50 had multiple findings. Among the 75 neonatally affected children, there were few obvious patterns to the findings. There were 21 children with both eyelid swelling and discharge. There were no children with both acne and natal teeth. There were 21 children with both nail deformities and eyelid swelling or discharge.

Among the transplacentally exposed children, logistic regression was used to examine the effects of certain factors on the rates of occurrence of these neonatal findings. Those children born shortly after direct exposure ceased were neither more nor less likely to exhibit the findings

TABLE 1. Neonatal dermatologic findings reported by parents

	Transplacentally exposed (%) $n = 128$	Control (%) $n = 115$	Older sibling (%) $n = 16$
Eyelid swelling	25 (20)	0 (0)	0 (0)
White eye discharge	32 (25)	5 (4)	0 (0)
Hyperpigmentation	54 (42)	2 (2)	1 (6)
Small/deformed nails	30 (23)	1 (1)	0 (0)
Acne	16 (13)	0 (0)	1 (6)
Natal teeth	13 (10)	0 (0)	0 (0)
Irritated/swollen gums	11 (9)	0 (0)	0 (0)
Any of above	67 (52)	7 (6)	1 (6)
Two or more of above	48 (38)	1 (1)	1 (6)

Items are from a general health questionnaire administered at initial interview in the home; however, the entry for natal teeth includes two children whose teeth were reported to the dentist rather than on the questionnaire. Entries are the number of positive answers (also expressed as a per cent); missing and 'don't know' answers are counted as negative answers. n, number of persons questioned.

shown in Table 1 than those born long afterwards ($P > 0{\cdot}10$). In addition, if a woman had several children born after exposure, the first of these was neither more nor less likely to have these problems than were later children ($P > 0{\cdot}10$).

Lifetime history as reported by parents

The results from the dermatology checklist, in which we asked whether various conditions had ever occurred are shown in Table 2. Note that we can readily compare the transplacentally exposed children only to their controls; their older siblings and mothers have had more time for the conditions to occur. The main differences between the transplacentally exposed and control children were deformities of the finger- and toe-nails. The mothers also reported acne scars more frequently for the exposed children. Generalized itching was reported more often for the transplacentally exposed children. Localized abscesses and warts were also reported more frequently in this group. However, the main finding was the 23 children with finger-nail deformities of whom 22 also had toe-nail deformities. The reported neonatal findings from the January questionnaire (Table 1) were compared with reported lifetime findings from the April questionnaire (Table 2); the latter are available only for those children who attended the clinic. Of the 16 children attending whose mothers had reported neonatal acne, only two were reported as ever having acne scars. Of the 28 children attending whose mothers had initially reported small or deformed nails at birth, only 18 were reported later as ever having had deformed finger- or toe-nails. The reason for this inconsistent reporting is not known; it is possible that 10 children had nails that were small but not deformed, but it is also possible that the reporting by the mothers was inaccurate.

Among the transplacentally exposed children, many of the reported lifetime findings were more prevalent in older children than in younger. This may be because children born soon after

TABLE 2. Lifetime history of dermatological findings reported by parents

	Transplacentally exposed (%) $n = 116$	Control (%) $n = 106$	Older sibling (%) $n = 15$	Exposed mother (%) $n = 56$	
Deformed finger-nails	23 (20)	0 (0)	0 (0)	21 (38)	$P < 0{\cdot}001$
Deformed toe-nails	31 (27)	1 (1)	1 (7)	19 (34)	$P < 0{\cdot}001$
Acne scars	11 (9)	0 (0)	2 (13)	35 (56)	$P < 0{\cdot}001$
Generalized itching	33 (28)	16 (15)	4 (27)	26 (46)	$P = 0{\cdot}023$
Allergy	13 (11)	10 (9)	1 (7)	5 (9)	$P > 0{\cdot}10$
Eczema	30 (26)	31 (29)	4 (27)	7 (13)	$P > 0{\cdot}10$
Abscesses or boils	26 (22)	11 (10)	4 (27)	15 (27)	$P = 0{\cdot}019$
Warts	8 (7)	1 (1)	2 (13)	7 (13)	$P = 0{\cdot}037$
Blistering in sun	5 (4)	1 (1)	0 (0)	2 (4)	$P > 0{\cdot}10$
Excess facial hair	4 (3)	1 (1)	1 (7)	1 (2)	$P > 0{\cdot}10$
Dark red urine	17 (15)	12 (11)	2 (13)	8 (14)	$P > 0{\cdot}10$
Hair loss	14 (12)	2 (2)	0 (0)	27 (48)	$P = 0{\cdot}004$
Numbness of hands or feet	1 (1)	1 (1)	1 (7)	36 (54)	$P > 0{\cdot}10$
Localized thickening of palms/soles	3 (3)	1 (1)	0 (0)	15 (27)	$P > 0{\cdot}10$

Items are from a checklist administered to attendees at the examination. Entries are the number of positive answers (also expressed as a per cent); missing and 'don't know' answers are counted as negative answers. n, number of persons questioned. The last column gives the significance level of Fisher's exact test comparing transplacentally exposed and controls.

the poisoning were more severely affected than those born later or because older children have simply had more time for the findings to occur. Logistic regression accounting for both age and sibling number (with the first child after the poisoning being sibling 1) showed that age was sometimes significant but sibling number was never significant ($P > 0{\cdot}10$ for all outcomes).

Physical examination

Hyperpigmentation persisted at the time of examination and occurred with a high prevalence in the children transplacentally exposed (Table 3). Post-traumatic and post-inflammatory pigmentation were excluded. Noticeable facial hyperpigmentation was three times more frequent in the exposed children than in controls ($P = 0{\cdot}045$, Fisher's exact test). Hyperpigmentation of the genitalia was more frequent ($P = 0{\cdot}0178$), with the rate higher in the transplacentally exposed than in their older siblings. Hyperpigmentation of the feet was also increased in the transplacentally exposed, although the increase was of borderline statistical significance ($P = 0{\cdot}0701$); rates in hands were unaffected ($P > 0{\cdot}10$). Rates of gingival hyperpigmentation were unaffected by transplacental exposure ($P > 0{\cdot}10$), but older siblings and exposed mothers showed high rates. The dentist who examined these children reported 43 transplacentally exposed, 33 controls and 11 siblings with oral pigmentation. For those examined after the first 3 days, a subjective assessment of general skin colour (light, moderate or dark) was noted; 11% of

TABLE 3. Results of dermatological examination

	Transplacentally exposed (%) $n = 117$	Control (%) $n = 106$	Older sibling (%) $n = 15$	Exposed mother (%) $n = 60$
Hyperpigmentation:				
Perineal/genital	50 (43)	29 (27)	3 (20)	0 (0)*
Face	13 (11)	4 (4)	3 (20)	36 (60)
Feet/toes	24 (21)	12 (11)	4 (27)	0 (0)*
Hands/fingers	9 (8)	5 (5)	2 (13)	0 (0)
Gingiva/lips	23 (20)	17 (16)	7 (47)	38 (63)
Finger-nails				
Dystrophic only	17 (15)	1 (1)	0 (0)	8 (13)
Pigmented only	0 (0)	0 (0)	1 (7)	20 (33)
Both	2 (2)	0 (0)	0 (0)	6 (10)
Toe-nails				
Dystrophic only	38 (32)	19 (18)	3 (20)	2 (3)*
Pigmented only	4 (3)	0 (0)	5 (33)	8 (13)*
Both	32 (27)	3 (3)	5 (33)	15 (25)*
Acne				
Acneiform lesions	11 (9)	7 (7)	1 (7)	39 (65)
Acne scars	11 (9)	6 (6)	6 (40)	46 (77)
Lesions and/or scars	20 (17)	10 (9)	7 (47)	50 (83)
Meibomian or conjunctival involvement	12 (10)	9 (8)	1 (7)	11 (18)
Scaly or keratotic disorders	31 (26)	17 (16)	8 (53)	7 (12)
Sweat gland disorders	7 (6)	3 (3)	0 (0)	0 (0)*

Entries are the number of persons with the condition (also expressed as a per cent). *n*, number of persons examined. * Indicates that mothers were often not examined.

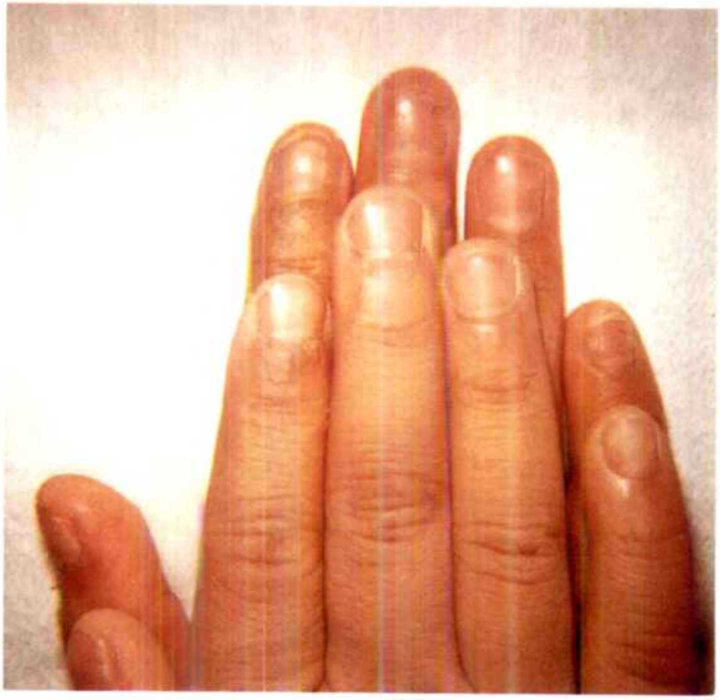

FIGURE 1. Pigmented and slightly dystrophic finger-nails of a 6 4/12-year-old male on bottom. Control child's nails on top.

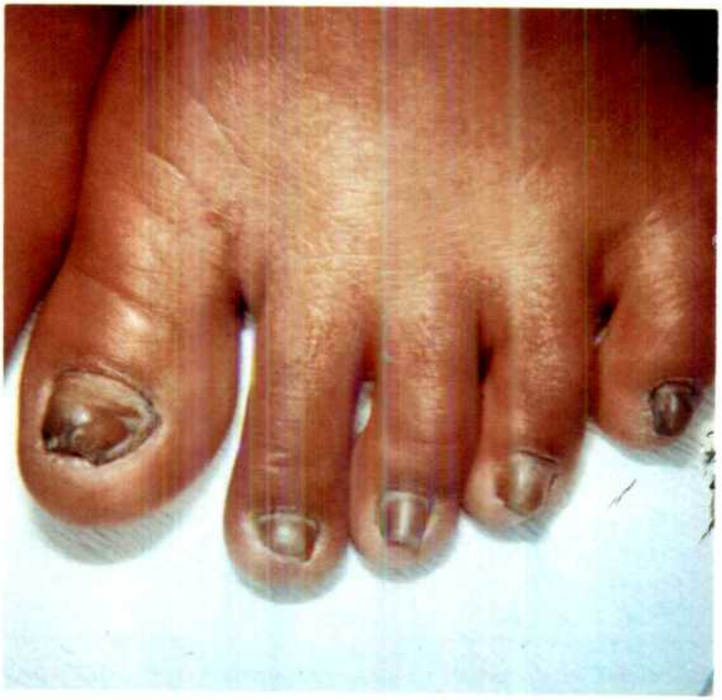

FIGURE 2. Pigmentation and onychodystrophy of toe-nails in a 5 9/12-year-old female. Note onychauxis of fifth toe-nail and increased transverse overcurvature of third toe-nail.

the transplacentally exposed were dark and 43% were light, as compared to 6 and 64%, respectively, in the controls. There was little tendency for general skin colour to be related to local hyperpigmentation. Only hyperpigmentation of the feet showed a relationship with general skin colour, and there it was stronger in the controls than in the exposed.

In the children dystrophic finger-nails (Fig. 1) essentially occurred only in those transplacentally exposed. Dystrophic toe-nails (Fig. 2) were less specific for they occurred in both the other groups of children, albeit much less often in the controls. Pigmented toe-nails were seen more commonly in the older siblings and less often in transplacentally exposed children. The exposed mothers had a high rate of pigmented finger-nails and toe-nails. Rates of dystrophic finger-nails, dystrophic toe-nails, and pigmented toe-nails were all statistically significantly higher in the transplacentally exposed than in the controls ($P < 0{\cdot}001$). The affected digits were often the first and fifth; 71% of all children with abnormal finger-nails and 61% with abnormal toe-nails had the abnormalities confined to these digits. The most common deformities seen in the finger-nails of children were grooves and ridges (including Beau's lines, other transverse grooves, ridges and ripples) and other unspecified dystrophy, affecting 10 children, and transverse overcurvature (pincer and tile-shaped nails) and longitudinal overcurvature (forward-curving and humped), also affecting 10 children. In the toe-nails, they were koilonychia (spooning) (37 children), ridging (33), onychauxis (thickening) (26), onychoschizia and other splitting/scaling (20), plication (flattening) (17), and transverse overcurvature (12). The exposed and control children who had nail abormalities had similar types of deformities except that the exposed had relatively more onychauxis and less plication. Of the 21 children with dystrophic or pigmented finger-nails, 18 also had dystrophic or pigmented toe-nails.

Acneiform lesions (acne, comedones, milia, folliculitis, follicular keratosis, follicular prominence) and/or scars at the time of the examination were twice as common in the transplacentally exposed children as in the controls ($P > 0{\cdot}10$); they were also seen in almost half of the older siblings. Of those with active lesions, seven of 11 exposed children, one of seven controls, no siblings, and five of 39 mothers had follicular keratoses. Other kinds of lesions showed no obvious link with exposure. Of those with either lesions and/or scars, 11 of 20 exposed, six of 10 controls, and all siblings had the problems confined to the face.

There were only two children with hypertrichosis (both in the forehead area) according to the dermatologists; both were transplacentally exposed. However, 18 children who were transplacentally exposed, five controls, and one of the siblings were considered separately to be hirsute. There were five children with verrucae, three who were transplacentally exposed and one each of the other groups. We expected large differences in granularity of the conjunctivae or persistent Meibomian gland dilatation; however, the three groups of children were about the same for these. The transplacentally exposed children had a higher frequency (not statistically significant, $P > 0{\cdot}10$) of scaly or keratotic disorders (hyperkeratosis, keratosis pilaris, ichthyosis, xerosis, lichenification, crusting, scaling, thickening, seborrhoea) than did the controls (Fig. 3); their older siblings had an even higher rate. The exposed children also had higher rates of sweat gland disorders (miliaria, sudamina, follicular papules), although the rates are low and the difference is again not statistically significant ($P > 0{\cdot}10$).

Of the 47 transplacentally exposed children with reported neonatal hyperpigmentation who were examined, 39 had some current hyperpigmentation; six had facial pigmentation, 27 genital, and 11 gingival hyperpigmentation. Of the 27 with neonatal small/deformed nails, 23 had current nail abnormalities. Of the 15 with neonatal acne, only three had current acneiform lesions and/or scars. The two controls with neonatal hyperpigmentation and the one with neonatal nail abnormalities no longer show them. The sibling with neonatal hyperpigmentation still had pigmentation in various sites and the one with neonatal acne showed no signs of acne.

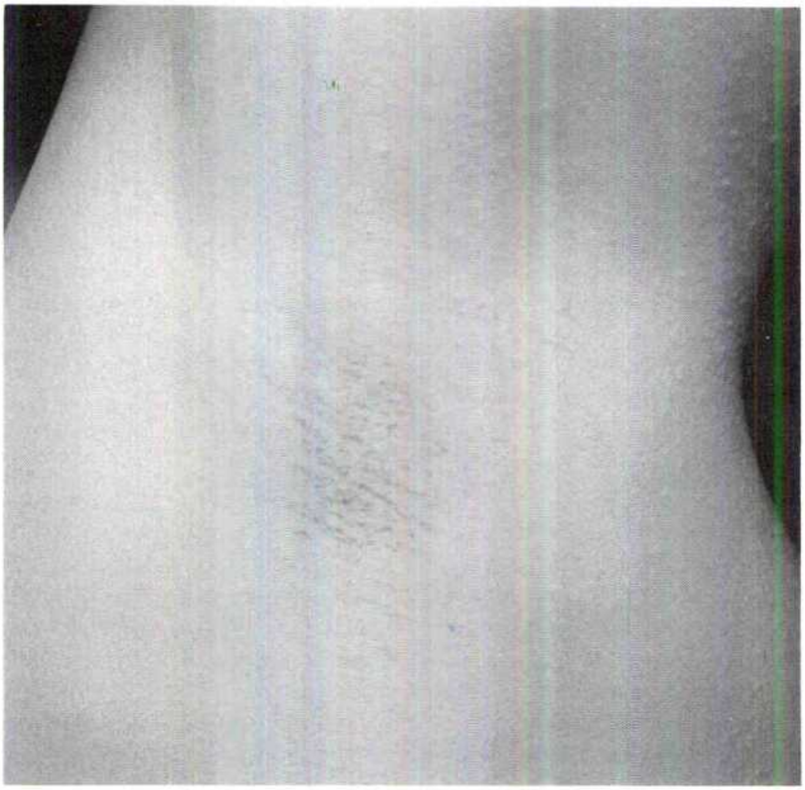

FIGURE 3. Axillary hyperkeratosis in a 6 10/12-year-old male.

DISCUSSION

In both this outbreak and in the epidemic in Japan, the skin was the major target organ. Although chloracne is regarded as a skin disease, there are various metabolic changes and signs of toxicity.[7,10,11] In these children, whose mode of exposure was different and who were exposed at a critical period of development, the dermatological findings are somewhat different from those seen in adults. The transplacentally exposed children appear to have an acquired neuro-ectodermal dysplasia with dental abnormalities, a growth deficit, developmental delay, and a behaviour disorder.[9] Although the chemicals are porphyrinogenic, an increased excretion of urinary porphyrins was uncommon but more frequent in the transplacentally exposed children.[12]

The primary findings in those directly exposed in the epidemic in Japan were acneiform eruptions and follicular hyperkeratosis, circumscribed hyperpigmentation and hypertrophy of the Meibomian gland.[5] The pigmentation was mainly on the nails, gingivae, face (especially nose), conjunctivae and lips. The main findings in the directly exposed patients in Taiwan were similar, but included xeroderma and keratotic plaques as well as deformed nails.[6] Those exposed to PCBs industrially have acneiform eruptions with skin and nail hyperpigmentation and eye symptoms are sometimes noted.[7,13,14]

The results here include some but not all of these disorders. Although acne was reported to be quite common at birth it did not persist. Three of the 15 children who were reported to have neonatal acne still had acne at examination. Many of the parents seen in 1985 still had active chloracne from their 1979 exposure, and this has been the experience in Japan and in industrial cohorts. About half of the older siblings with potential direct exposure that we saw had acneiform lesions and/or scars, compared to only 17% of the transplacentally exposed children. This observation calls into question the concept that chloracne is the most sensitive biological indicator of significant exposure to this class of compounds. Nail changes were a much more striking indicator in these children than was acne. Another difference from direct exposure is the distribution of hyperpigmentation. In our group the finger-nails, gingiva/lips, and conjunctivae were not affected, but the genitals, face and feet were. Gingival hyperpigmentation was seen at much lower levels than in those directly exposed and conjunctival hyperpigmentation was never seen. The Meibomian gland was unaffected by exposure, although mothers reported swelling and discharge of the eyes in their newborn infants.

The nail changes were striking and significant. The nail pigmentation appears to involve both the nail plate and bed. Nail pigmentation has been reported in PCB-exposed workers.[13,14] Nail elevation and loss occurs in rhesus macaques after intake of PCB isomers, dioxins and furans.[15] The nail changes were the first to occur in adult animals and were also seen in babies of nursing females fed non-fetotoxic doses of PCB isomers (W.P. McNulty, personal communication). X-rays of the hand were not done in our study or in the other exposed Taiwanese.

There is little doubt that chemical exposure during gestation produced these abnormalities. The polychlorinated biphenyls that were the major contaminants of the cooking oil ingested by these children's mothers are mixtures of over 200 distinct congeners. The PCBs were heat-degraded during use and thus were themselves contaminated by the PCDFs and by polychlorinated quaterphenyls.[2,3] Workers and offspring of workers exposed to PCBs that are relatively contaminant-free are not so severely affected, even though they have had exposures that produce blood concentrations of PCBs similar to those seen in these parents.[13] There has not been a human exposure to PCDFs alone. We can say only that the mixture is toxic, but there is strong suspicion that the PCDFs play the major role in its toxicity. Although it is clear that some of these chemicals cross the placenta,[16–18] exactly which is not known.

PCBs are among the most dispersed and persistent of environmental pollutant chemicals, and exposure to relatively low background amounts is ubiquitous.[19,20] PCBs, and to a lesser extent PCDFs, are a public health problem.[21]

The findings in these children are also an example of a rarely reported but potentially widespread phenomenen. While there are many transplacental toxins, there are few other known examples of transplacental dermatotoxins. Minoxidil, a drug with known trichogenic properties, apparently produced hypertrichosis in a child exposed transplacentally.[22] Phenytoin is associated with a syndrome of diverse structural anomalies; nail pigmentation and nail and/or distal phalangeal hypoplasia[23] and neonatal acne[24] have each been reported in a child transplacentally exposed. Carbamazepine, another anti-convulsant, may have produced nail hypoplasia in another child.[25] Although it may be a coincidence, phenytoin and carbamazepine are both inducers of mixed function oxidase enzymes, as are PCBs. There are reports of five cases of cutis laxa (and varying other defects) associated with penicillamine.[26] Hexachlorobenzene produces porphyria in adults and *pembe yara* (a disease characterized by pink sores and producing high mortality) in babies,[27] but the important exposure appears to be through breast milk. Thus, these findings represent a rarely described phenomenon and indicate the possibility of transplacental origins of other dermatological problems.

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Inhibition of photosensitivity in erythropoietic protoporphyria with terfenadine

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SUMMARY

The effect of terfenadine on the response to irradiation with blue light was measured in seven patients with erythropoietic protoporphyria. Terfenadine caused significant inhibition of the immediate flare reaction, but did not alter the erythemal response localized to the irradiation sites. Treatment with β-carotene had no effect on the flare and erythemal responses. However, the flare reaction was still inhibited by terfenadine during treatment with β-carotene.

These results show that histamine release is involved in the mechanism of porphyrin photosensitivity. H_1 receptor antagonists may be of use in the treatment of patients with erythropoietic protoporphyria.

Erythropoietic protoporphyria (EPP) is an inherited, photosensitive disorder, first described by Magnus *et al.* in 1961,[1] in which decreased activity of the enzyme ferrochelatase leads to increased blood levels of free protoporphyrin. Exposure to visible light, especially wavelengths around 400–450 nm (blue light), causes excitation of the protoporphyrin molecule and results in tissue damage, probably through generation of active oxygen species. This is manifest clinically as a burning or stinging sensation occurring within minutes of exposure to bright sunlight. The exposed skin may become red and oedematous and eventually becomes thickened, especially over the dorsum of the hands. Superficial pitted and linear scars are often seen on the cheeks and nose.[2]

Conventional treatment is with β-carotene, given orally, which, in the majority of patients provides some degree of photoprotection,[3] probably as a result of its ability to quench free oxygen species.[4] Other inflammatory mechanisms may be involved as mast-cell degranulation has been reported in EPP[5] and irradiation of cultured murine mast cells in the presence of protoporphyrin causes release of preformed mediators.[6]

The acute symptoms of photosensitivity in EPP are similar to those of solar urticaria, an idiopathic photosensitivity disorder in which sun exposure causes mast cell degranulation.[7] We have shown that terfenadine, a potent H_1 receptor antagonist, inhibits the photosensitive

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reaction in solar urticaria.[8] The effect of terfenadine on the cutaneous response to irradiation with blue light in patients with EPP, both before and during treatment with β-carotene is now reported.

METHODS

Effect of terfenadine in EPP

Patients. Seven male patients aged between 9 and 43 years (median 13 years) were studied. The age at onset of photosensitivity ranged from 1 to 5 years and the diagnosis was confirmed in each case by quantitative analysis of blood protoporphyrin levels.

Light source and phototesting procedure. The light source was a 1 kW high-pressure mercury lamp doped with iron and gallium iodides to enhance the spectral emission in the range 350–450 nm. The radiation passed through optical filters to isolate only blue light (400–470 nm; Fig. 1) and was focused into a light guide to produce a uniform beam (10 mm diameter) on the skin surface with an irradiance of 130 mW/cm^2.

Six sites, 10 mm diameter, on one side of the back of each patient were irradiated consecutively using a geometrical series of increasing exposures (dose increment factor 2). The maximum exposure dose was 48 J/cm^2. Five minutes after completing each irradiation, the maximum diameter of any flare produced at the sites was measured. One hour after irradiation the intensity of erythema at each of the sites was measured using a reflectance instrument[9] as described previously.[10]

The irradiation procedure and measurements of flare and erythema were repeated on the other side of the back after each patient had been treated with terfenadine (60 mg b.d. < 12 years; 120 mg b.d. > 12 years) for 48 h.

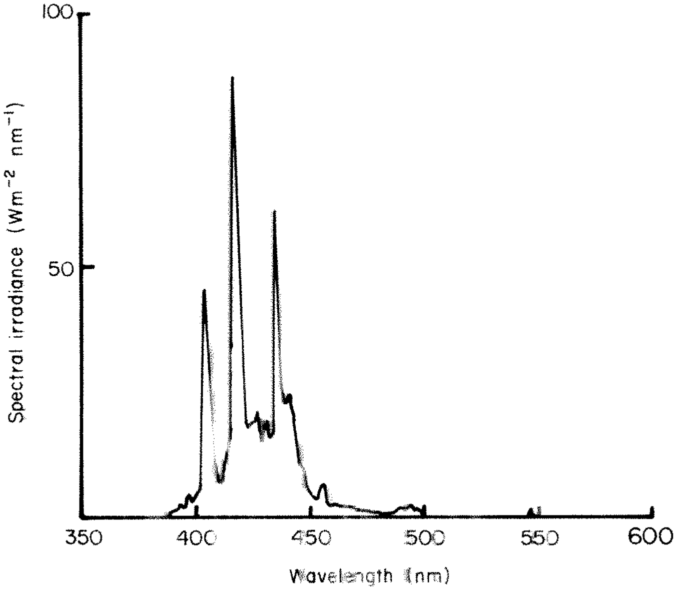

FIGURE 1. The spectral irradiance in the plane of the patients' skin.

Effect of terfenadine in EPP during treatment with beta-carotene

Five of the seven patients studied previously were treated for at least 2 months with β-carotene, given orally (30–90 mg daily). For each patient the dose of β-carotene given had resulted in serum levels within the therapeutic range during previous years of treatment. Serum levels of β-carotene were measured prior to phototesting using the light source and irradiation protocol described above. The patients were then treated with terfenadine (60 mg b.d. < 12 years; 120 mg b.d. > 12 years) for 48 h in addition to the β-carotene. The phototests were repeated and measurements of flare and erythema made as before.

Statistics

For the analyses comparing the effect of terfenadine or β-carotene on flare diameter across the dose range, the following procedure was used. For each patient the difference in response at each dose was calculated; a sign test was performed on the differences for each patient and the one-sided *P*-value calculated. The *P*-values were aggregated over patients by calculating the sum, S, of the values of $-2\log_e P$.[11] If terfenadine has no effect, then theory suggests that this sum is distributed as χ^2; this follows because each *P*-value is distributed uniformly between 0 and 1. As only a few *P*-values can actually be achieved, 1000 random samples were drawn from the population of possible *P*-values and S calculated for each. The significance level of the observed S was found by referring it to this randomization distribution, an approach similar to that described by Farr *et al.*[12]

For other comparisons the Wilcoxon signed-rank test was used.

RESULTS

Effect of terfenadine in EPP

In each patient, erythema, localized to the irradiation sites, developed during irradiation and persisted for several hours. By the end of irradiation, an irregularly bordered flare that extended

TABLE 1. The increase in erythema index[10] compared with unirradiated control skin 1 h after irradiation with 48 J/cm^2 blue light before and during treatment with terfenadine and β-carotene. Values are means of triplicate of measurements: a typical standard deviation is 0·008. (Values around 0·050 are equivalent to minimal erythema; values around 0·100 are equivalent to erythema of moderate intensity).

Patient number	No treatment	During treatment with terfenadine	During treatment with β-carotene	During treatment with terfenadine and β-carotene
1	0·095	0·071	0·083	0·067
2	0·102	0·059	0·094	0·124
3	0·103	0·095	0·073	0·086
4	0·102	0·082	0·112	0·109
5	0·101	0·091	0·083	0·114
6	0·076	0·114		
7	0·077	0·091		

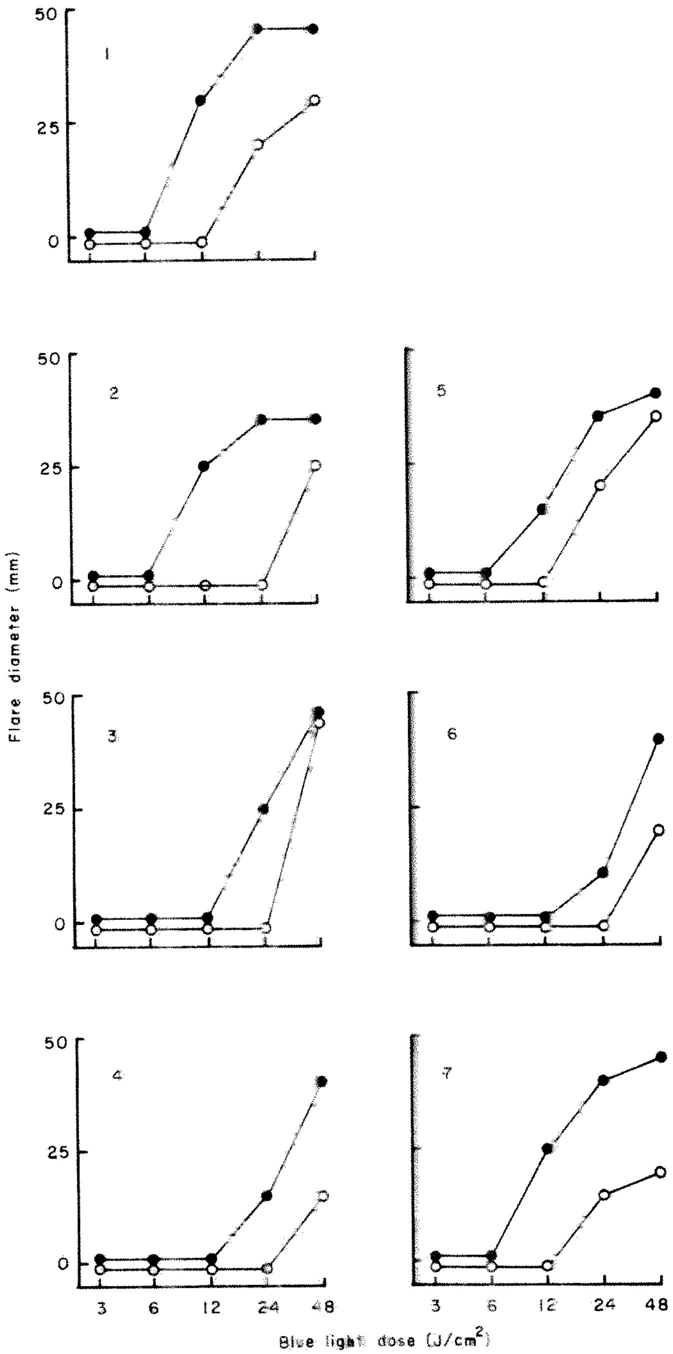

FIGURE 2. The diameter of the flare produced by different doses of blue light in each of the seven patients before (●) and during treatment (○) with terfenadine.

beyond the sites was seen in all patients. This became maximal at about 5 min and had resolved within 30 min. The diameter of the flare increased with increasing doses of light (Fig. 2). Treatment with terfenadine significantly increased the smallest dose of light required to produce a flare (median increase 18 J/cm,^{2} $P=0{\cdot}022$, Wilcoxon signed-rank test; 95% confidence interval [12, 30]). Terfenadine decreased the maximum flare diameter across the dose range ($P=0{\cdot}001$, randomization test).

During treatment with terfenadine, there was no significant change in the intensity of erythema at the irradiation sites, measured 1 h after irradiation (Table 1; median decrease in erythema index 0·01, $P=0{\cdot}45$, Wilcoxon signed-rank test; 95% confidence interval [$-0{\cdot}034$, 0·026]).

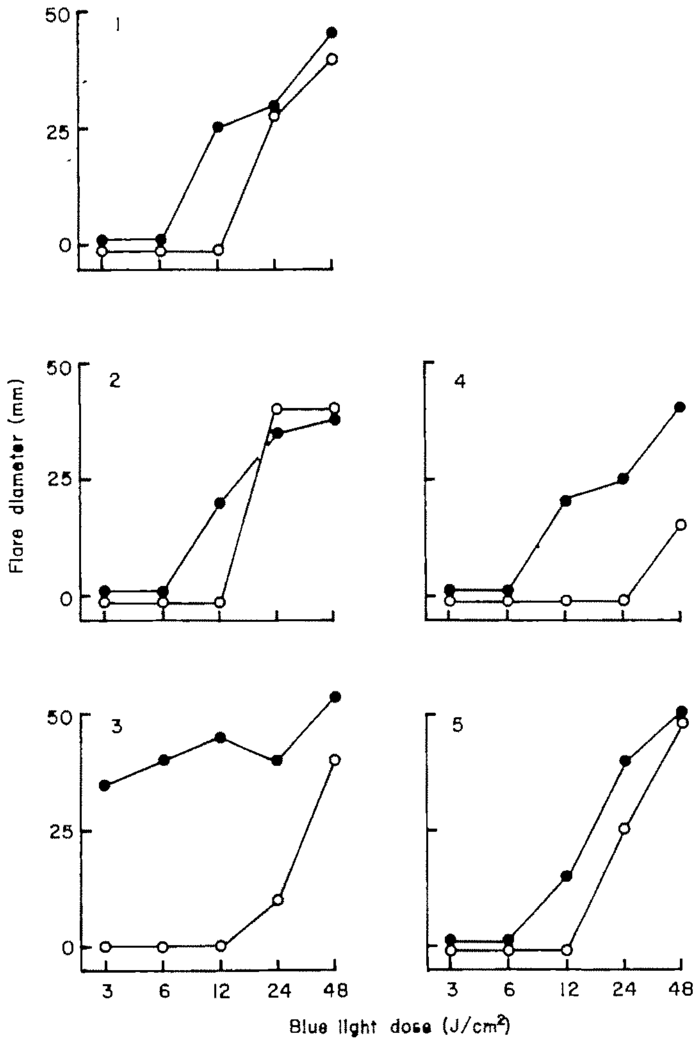

FIGURE 3. The diameter of the flare produced by different doses of blue light in each of the five patients during treatment with β-carotene, before (●) and during treatment (○) with terfenadine.

Effect of terfenadine in EPP during treatment with β-carotene

At the time of phototesting the β-carotene level in each patient was within the therapeutic range ($> 7·4$ μmol/l).[13] During treatment with β-carotene, the flare reaction (but not erythema) was inhibited by terfenadine ($P = 0·004$, randomization test) (Fig. 3; Table 1). Using the randomization test, there was no evidence of a difference between the flare diameters on and off β-carotene, before terfenadine was used ($P = 0·59$), nor was there a difference between the effect of terfenadine before and during treatment with β-carotene ($P = 0·24$). Likewise, there was no change in erythema during treatment with β-carotene compared with pretreatment values ($P = 0·75$) (Table 1).

DISCUSSION

It has been shown that treatment with terfenadine, a potent H_1 receptor antagonist, significantly inhibits the flare response to blue light in patients with EPP who are on no other treatment, and in those treated with β-carotene.

In normal subjects, exposure to a dose of blue light of 24–48 J/cm^2 at this irradiance (130 mW/cm^2) results only in erythema, which is localized to the irradiation site, develops during irradiation and resolves within 1 h (P. M. Farr, unpublished data). This erythema is probably a thermal response to the high rate (130 mW/cm^2) of energy deposition in the skin. In the patients, with EPP, erythema occurred at lower doses and persisted for up to 24 h. The intensity of erythema localized to the irradiation sites was not reduced during treatment with terfenadine, showing that this component of the inflammatory reponse to blue light in EPP is not mediated by histamine acting on the H_1 receptor.

The irregularly bordered flare that developed at sites exposed to higher doses of blue light is similar to that seen in solar urticaria,[8] a disorder in which mast-cell degranulation and histamine release occur.[7] This flare was inhibited by terfenadine and indicates that part of the inflammatory response to blue light in patients with EPP is mediated by histamine acting on the H_1 receptor.

The mechanism of porphyrin photosensitivity is thought to be due to generation of free oxygen species[14] and possibly through activation of complement.[15] Our data show that histamine release is a further mechanism of porphyrin photosensitivity in EPP. A previous study found no change in skin reaction to 400 nm radiation in five patients with EPP treated with H_1 receptor antagonists such as chlorpheniramine and mepyramine maleate.[16] One explanation for these findings is the lower potency of conventional H_1 receptor antagonists, compared with terfenadine.[17-19]. It is of interest that in mice injected with haematoporphyrin derivative, irradiation with visible light has been shown to cause histamine release.[20] Our findings may therefore also be of relevance to the persistent photosensitive reaction induced by haematoporphyrin derivative which is increasingly used for cancer therapy.[21]

EPP is usually treated with β-carotene and we re-tested the five patients who were being treated with this drug. Despite achieving blood levels of β-carotene in excess of that recommended for maximum effect,[13] we were unable to demonstrate any change in flare or erythemal response compared with pre-β-carotene values. This suggests that any photoprotective effective of β-carotene in EPP is not mediated through inhibition of immediate flare response or erythemal response up to 1 h after exposure. The number of patients we tested was small and a larger study will be needed to exclude a small effect, although a previous study also found no consistent change in erythemal response (assessed as minimal response dose immediately after irradiation, at 7–8 h, 24 h and 48 h) using various wavelengths from 400 to 600 nm in 19 patients with EPP treated with β-carotene.[22]

We have shown that treatment with terfenadine inhibits the immediate flare reaction to blue light in patients with EPP and that this effect persists during conventional treatment with β-carotene. Beta-carotene does not provide complete protection against the effects of sun exposure in EPP[3] and evidence of its therapeutic effect remains anecdotal rather than based on controlled clinical trials. H_1 receptor blockade may provide further protection against sunlight in this rare photosensitivity disorder.

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Trial of nedocromil sodium in atopic eczema

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SUMMARY

The efficacy of orally administered nedocromil sodium, a mast-cell stabilizer, was assessed in the treatment of adults with atopic eczema plus food allergy. Twenty adults with atopic eczema and allergy to hens' eggs, cows' milk or wheat took part in this double-blind, cross-over study of nedocromil sodium versus placebo, each treatment period lasting 4 weeks. No significant differences were found between the two treatments on clinical assessment, or by measurement of total and specific IgE levels.

Approximately 25% of adults with severe atopic eczema have associated IgE-mediated food allergy.[1] Although in some patients this only causes angioedema, in others it may be associated with delayed exacerbation of the eczema over the following 2–24 h. Patients can usually easily avoid allergens such as fish or nuts, but those allergic to common foods such as hens' eggs, cows' milk or wheat may unwittingly be ingesting small amounts in prepared foods such as sauces or confectionery.

The rationale behind the use of orally administered mast-cell stabilizers in atopic eczema associated with food allergy is that, by stabilizing the small intestinal mucosal mast cells, inflammation precipitated by the presence of an allergen is prevented, and this results in its decreased absorption. Previous studies with the mast-cell stabilizer sodium cromoglycate have proved disappointing.[2] Nedocromil sodium has been shown to be markedly more active *in vitro* than sodium cromoglycate in stabilizing mucosal mast cells.[3] It has been shown, *in vivo*, to be as effective as sodium cromoglycate in asthma[4,5] and in suppressing allergic conjunctivitis when instilled into the eye.[6]

In this study we assessed the efficacy of orally administered nedocromil sodium in the management of patients with atopic eczema associated with IgE-mediated food allergy.

METHODS

Adults between the ages of 16 and 65 years with moderate or severe atopic dermatitis were chosen for the study, and all had a history of allergy to the common foods, hens' eggs, cows' milk

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or wheat, confirmed by positive skin-prick test (>5 mm weal) or by a strongly positive radioallergosorbent test (3 or 4). Patients on regular oral steroid therapy were excluded. It was a double-blind, placebo-controlled cross-over study lasting 4 months. After a 4-week baseline period the patients were randomly assigned to nedocromil sodium 100 mg three times daily or to placebo (matching dextrose tablets) for 4 weeks. At the end of this period they were switched to the alternative treatment. The trial was completed after a 4-week follow up. Throughout the study patients continued on their normal diet which was assessed by a dietitian to see whether food allergens were totally, partially or not excluded. All patients were monitored at monthly intervals using a grading system 0–6 to record the severity of the eczema, and a modification of the rule of nines system was used to find the extent of body involvement. Patients also kept a daily diary card to record symptoms of itching, redness and weeping of the skin using a 0–10 linear analogue scale. Topical therapy was standardized to betamethasone valerate 0·02% and 1% hydrocortisone and the amount used was weighed for each 1-month period. Full blood counts and tests of renal and hepatic function were performed each month to determine any drug toxicity.

The scores were compared for the final 2 weeks of each 4-week period in order to minimize any possible carry-over effect. The results were analysed using a non-parametric analysis for cross-over trials and a parametric analysis of variance with a factor for order, period and treatment.

RESULTS

Twenty patients (six male, 14 female; age range 17–61 years, median 29 years) completed the study. Two additional women were enrolled but proceeded no further than their first visit; one failed to return and the other became pregnant.

All patients were food allergic, hens' eggs being the commonest allergen (16 patients), then fish (6), cows' milk (6), pork (2) and wheat (1). Dietary assessment demonstrated that six patients totally avoided foods to which they were known to be allergic, 12 patients partially avoided and two did not avoid the allergens.

Compliance was good with only one patient withdrawing early while on placebo because of uncontrolled eczema, and two on nedocromil sodium, one because of uncontrolled eczema and the other because of persistent diarrhoea.

Analysis of the clinician's overall opinion of the severity of the eczema and the body chart

TABLE 1. Parametric analysis of scores for body chart symptoms

	Mean score for				Nedocromil – placebo	
Variable	Baseline	Nedocromil	Placebo	Follow-up	Mean (95% confidence interval)	P value
Overall severity	3·13	2·87	3·09	2·78	−0·22 (−0·71–0·26)	0·33
Erythema	2·69	2·58	2·51	2·28	+0·07 (−0·33–0·46)	0·73
Excoriation	2·09	1·81	1·97	1·62	−0·16 (−0·50–0·18)	0·34
Dryness	1·42	1·37	1·38	1·16	−0·01 (−0·47–0·45)	0·96
Proportion body involved	0·41	0·38	0·38	0·40	0 (−0·07–0·07)	0·97

Symptom scale: 0, none; 1, minimal; 2, mild; 3, moderate; 4, moderate–severe; 5, severe; 6, very severe.

scores (Table 1), showed no significant differences between active and placebo treatments. Neither the non-parametric analysis nor the parametric analysis of variance of cross-over data showed any significant differences; the latter is presented in Table 1. Period and order effects were not statistically significant for any variable. The patient's preference for treatment was for nedocromil sodium in seven cases, placebo in nine and neither in four. The diary card symptom scores, topical steroid usage and total and specific IgE levels showed no significant differences between active and placebo treatments. No abnormalities were found in laboratory data, and the drug was well tolerated apart from one patient who developed persistent diarrhoea, which ceased on withdrawal of the drug.

DISCUSSION

The pathogenesis of atopic eczema is not fully understood, but it seems likely that IgE-mediated allergy plays an exacerbating rather than intrinsic role in the disease. Patients with atopic eczema and food allergies describe a variety of reactions to food allergens. Immediate angioedema and urticaria may be the only symptoms, but this is often associated with a delayed aggravation of eczema or increased itching,[7] possibly due to late phase leukotriene release.

As only 3% of nedocromil sodium is absorbed into the bloodstream, the drug ingested orally would be expected to have its main effect in preventing inflammation in the small intestine, thereby suppressing absorption of food allergens. This in turn may prevent binding of the allergens to IgE on skin mast cells with consequent degranulation of these cells, release of inflammatory mediators and exacerbation of the eczema. It has been shown that patients with atopic eczema tend to have increased permeability of the small intestine to inert sugar molecules.[8,9] We have shown that patients with atopic eczema and proven IgE-mediated food allergy have circulating precipitating antibodies (mainly IgG and IgA) to common food allergens.[10] It has been presumed that such antibodies are formed due to increased absorption of foods, as is the case in patients with coeliac disease, who have similar circulating precipitating antibodies[11] and increased gut permeability.

In this study nedocromil sodium administered orally appears to have had no beneficial effects in the management of patients with atopic eczema. This was the case both in patients who completely avoided incriminated foods, and those who only partially avoided them. This suggests that in adults, either allergy to common foods is not one of the major factors influencing the exacerbation of eczema, or alternatively sufficient allergen absorption may be continuing either via the buccal mucosa where the nedocromil has not been dispersed and is therefore not active, or via the small intestine because of the failure of the nedocromil to stabilize mucosal mast cells.

In view of the success of 'topically' administered preparations to the lung (by inhalation) and the eye, it will be of interest to see whether nedocromil sodium applied topically to the skin has any effect in suppressing exacerbations of atopic eczema. However, we conclude that oral nedocromil sodium has no part to play in the management of atopic eczema in adults.

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Rothmund–Thomson syndrome: a report of two patients and a review of the literature

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SUMMARY

Two siblings with poikiloderma, short stature and other abnormalities are described. They were originally reported as having Morquio syndrome; subsequently, mucopolysaccharidosis was excluded biochemically and they were classified as 'a newly recognized syndrome of connective tissue dysplasia'. It is now clear that their disorder is the Rothmund–Thomson syndrome (RTS).

A brother and sister with poikiloderma, short stature and other musculoskeletal abnormalities were found at ages 3 and 6 to have increased urinary excretion of mucopolysaccharides and were reported as having Morquio syndrome.[1] As teenagers they were reinvestigated because they had not developed the expected corneal and spinal changes. There was no longer any evidence of mucopolysaccharidosis and their disorder could not be classified.[2] They were reviewed aged 25 and 27 when additional abnormalities had developed and the clinical picture was suggestive of Rothmund–Thomson syndrome (RTS).

CASE REPORTS

Both patients were described in detail during early childhood[1] and as teenagers,[2] and were presented at a recent dermatological meeting.[3]

Patient 1

This male presented at the age of 3 months with a urinary tract infection, and at 1 year with growth retardation, kyphoscoliosis, and telangiectasia and atrophy of the skin of the face and distal limbs. During childhood he required surgery for chronic bilateral suppurative otitis media. He developed increasing spasticity of the legs with severe talipes of the left foot, attributed to his spinal deformity. By the age of 15 he had bilateral iris atrophy. At the age of 21 hypertension was first noted and 2 years later he developed hypertensive encephalopathy and a hemiparesis from which he recovered following control of his blood pressure. At that time

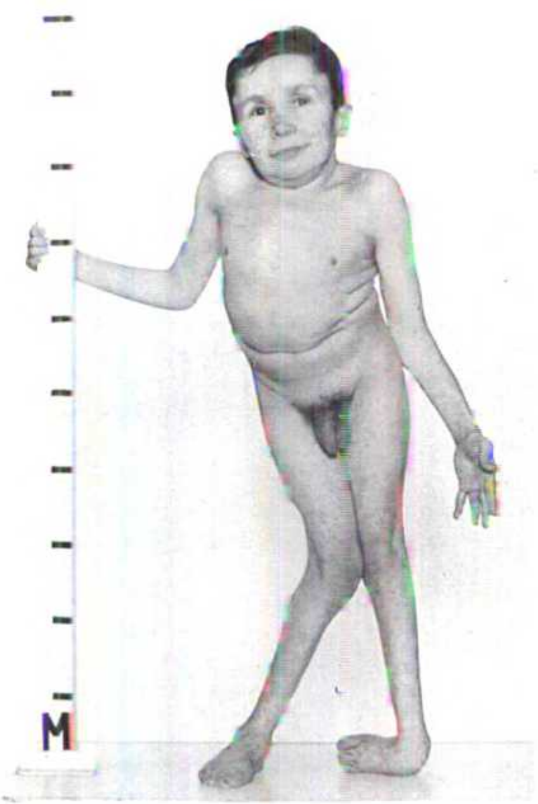

FIGURE 1. Patient 1, showing short stature, kyphoscoliosis, genu valgum, equinus deformity and poikiloderma on face and extremities.

ophthalmological examination revealed bilateral band keratopathy and anterior and posterior cataracts.

He is now 25 years old and his height is 97 cm and his weight 15 kg (Fig. 1). He is proportionately dwarfed except for excessive spinal shortening due to curvature. He has marked telangiectasia on the face and the extensor surfaces of the limbs, most marked on the hands, with wrinkling of the affected skin. He is not photosensitive. He has chronic painful hyperkeratotic ulcers on the telangiectatic skin, which leave punctate atrophic scars. There is a café-au-lait macule on the left shoulder. He has a saddle-nose (Fig. 2), severe kyphoscoliosis, genu valgum, and equinus deformity of the left foot. Joint mobility is increased at the wrists and fingers, and decreased in the shoulders, knees and elbows, which show fixed flexion. He appears hypogonadal with an unbroken voice, little facial hair and no axillary hair, although the pubic hair and external genitalia appear normal. Scalp hair, eyebrows and lashes are normal with no greying. The fingernails are poorly developed with increased longitudinal ridging; his teeth are normal.

Ophthalmological examination shows a visual acuity of 6/36 (right) and 6/24 (left), a grossly deficient anterior mesodermal layer of the iris, numerous sphincter transillumination defects, and a left fixed dilated pupil. There are bilateral nuclear and posterior cortical lens opacities

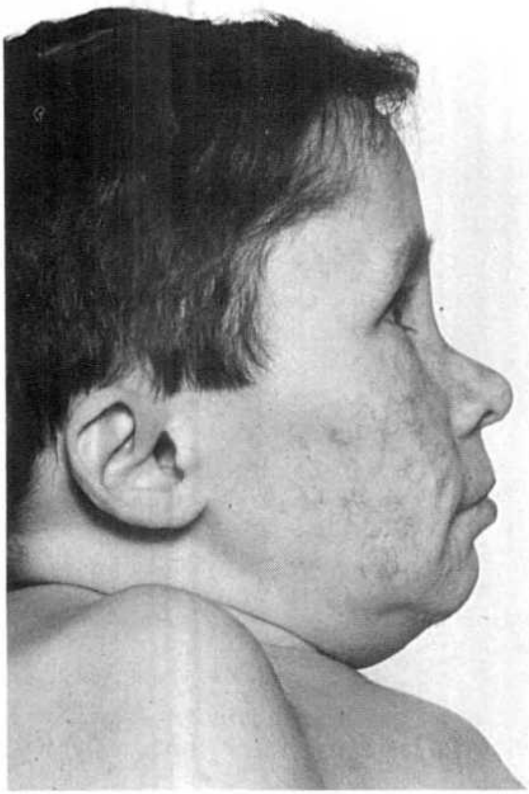

FIGURE 2. Patient 1 showing saddle-nose, sparse facial hair, telangiectasia and punctate scars.

precluding a clear view of the fundus, which had previously shown retinitis punctata albescens. He is of normal intelligence.

Investigations. At the age of 3, the 24 hour urinary excretion of mucopolysaccharides was increased.[1] The mucopolysaccharides were not characterized. At the age of 15 there was no excess mucopolysacchariduria and tests excluded all the mucopolysaccharidoses, mucolipidoses and sphingolipidoses.[2] Collagen types I, III, and VI in skin were normal (Dr M.Pope, Northwick Park Hospital).

Radiological abnormalities at the age of 15[2] included dolichocephaly, mid-face hypoplasia, gross mid-thoracic kyphoscoliosis, elongated bodies of the lumbar vertebrae with irregular concave endplates, some abnormalities of the pelvis, flattening of the capital epiphyses of the femora, short femoral necks, undertubulation of the metaphyses of the long bones of the legs with longitudinal streaks of sclerosis immediately proximal to the epiphyses, upward subluxation of the humoral heads in poorly developed glenoid cavities, shallow olecranon fossae with posterior dislocation of the radial heads, and sclerosis of the distal radial metaphyses. There was generally delayed skeletal maturation (bone age 8·1 years at chronological age 15 years). The endocrine evaluation was normal at age 15 (testosterone 11 nmol/l) but it has not been repeated since puberty.

Blood tests at the age of 15 revealed an elevated urea of 12·2 mmol/l. Other biochemical tests, blood count, electrocardiogram and echocardiogram were normal. Investigations 8 years later showed chronic renal failure with blood urea 22 mmol/l, creatinine 141 μmol/l, creatinine clearance 11 ml/min, 24-h urinary protein excretion 0·7 g, and normal C3, C4 and immunoglobulins apart from raised IgA of 3·11 g/l. An ECG showed left ventricular hypertrophy; the urine was sterile and contained no cells; IVP showed a normal right kidney and parenchymal loss and calyceal dilatation at the left upper pole; renogram showed no renal artery stenosis; ultrasound showed no coarctation of the aorta; catecholamine excretion was normal. It was concluded that his hypertension was secondary to chronic pyelonephritis. He has a normal 46XY karyotype with no increase in sister chromatid exchange rate. Urinary amino acids and dipeptides are normal with no evidence of prolidase deficiency.

Patient 2

This female developed telangiectasia of the face and limbs during her first year. At 14 months she was found to have bilateral dislocated hips and to be underweight. Her problems since then have been a short stature, pain in the hips and elbows, and skin ulcers. Menstruation commenced at 15·5 years but was always sparse and irregular. She was sterilized by tubal ligation at age 22. When last examined at age 27 her height was 125 cm and weight 40 kg. All parts of her body were reduced proportionately. The skeletal abnormalities were saddle-nose, genu valgum, hyperextensible fingers and wrists and reduced mobility of elbows and hips. General and neurological examination were normal and she was of average intelligence. She had telangiectases on the face, arms, hands and lower legs, especially the extensor aspects. The affected skin, especially on the backs of the hands, was fine and wrinkled, and faintly pigmented. Scalp hair was fine and body hair sparse. The nails were hypotrophic with increased longitudinal ridging. Ophthalmological examination revealed a visual acuity of 6/9 in the right eye and 6/6 in the left. The cornea was normal, with a diameter of 10 mm. Schwalbe's line was not unduly prominent, but there was iris atrophy. There were no cataracts. She had tilted discs with peripapillary atrophy.

Investigations. At the age of 6, 24 h urinary excretion of mucopolysaccharides was increased,[1] but when aged 17 extensive studies showed no biochemical abnormality.[2] Radiological abnormalities at the age of 17[2] were similar to but milder than her brother's and included moderate dolichocephaly, mid-face hypoplasia, and an abnormally straight spine with long narrow vertebrae. The pelvis was normal apart from acetabular displacement, flattening of both femoral heads, and short femoral necks with coxa vara deformities. The glenoid cavities were poorly developed and the radial heads deficient and dislocated backwards. There was sclerosis of the inferior metaphyseal region of both femora. Endocrine tests were normal apart from a rather protracted LHRH response and marginal impairment of thyroid responses. Other biochemical, haematological and immunological tests were normal apart from a raised IgA (7·04 g/l). Collagen types I, III and VI were normal in skin (Dr M Pope, Northwick Park Hospital). She has a normal 46XX karyotype with no increase in sister chromatid exchange rate. A biopsy of a chronic ulcer showed a mixed inflammatory infiltrate in the dermis, some regenerative activity, and acanthosis and hyperkeratosis of the surrounding epidermis. Urinary amino acids and dipeptides are normal, with no evidence of prolidase deficiency. Endocrine evaluation at age 27 showed normal basal prolactin, LH and FSH levels, but a low oestrogen and impaired LH response to LHRH. Twenty-four hour urinary cortisol excretion was low although the 09.00 level was normal. Free thyroxine index was 18·8 (normal 21–36) and T4 level 67 nmol/l (normal 63–131 nmol/l), while TSH was 6·6 mU/l (normally less than 6·0 mU/l).

Family history

These two siblings are the only affected family members, with a normal older sister and no history of consanguinity.

DISCUSSION

The abnormalities found in these patients have previously been reported in Rothmund–Thomson syndrome (RTS), a rare autosomal recessive disorder, of which 119 other cases are reported in the literature. Several individual patients with RTS have been reported by different specialists at different times,[4] and these 119 cases are derived from some 140 separate case reports.[5–62]

The characteristic poikiloderma is never congenital but develops in the first year of life, particularly affecting the cheeks and extensor surfaces of the hands, forearms and legs. The affected skin is soft and pliable, an impression which contributed to the earlier diagnoses of Morquio syndrome and connective tissue disorder. Multiple discrete round scars, and persistent infection under a hyperkeratotic crust are well described,[11] but recurrent ulceration has not previously been reported. Hyperkeratotic lesions on the extremities become more frequent with age, occurring in 13% of patients under 12 years and 27% of patients aged 12 years or more. Low blood concentrations of vitamin A have been found in patients with dyskeratoses,[7] and in one case the lesions improved with retinoids.[60] The hyperkeratotic lesions may undergo Bowenoid change[60,38] and are considered premalignant.[60] However, there are only four reports of squamous carcinoma of the skin in RTS[10,11,45] and it is not certain that these all developed in pre-existing verrucous lesions.

Photosensitivity, reported in 17 patients, is more common in early childhood and usually resolves,[7,53] as in our patients, although Berg *et al.*[59] described a 25-year-old woman with photosensitivity clinically to sunlight and experimentally to UVA.

The nail dystrophy reported in 20 out of 120 patients is most commonly hypertrophy and slow growth as in our patients,[10,11,17,18,22,28,40] but onycholysis[52] and nail hypertrophy[5,20] have also been described. A wide variety of dental anomalies is reported.[22,32] Fifty-seven reported cases had sparse hair, affecting the scalp in 42 and eyebrows and lashes in 41. Frequently, facial, axillary and pubic hair is reduced, and biopsies of poikilodermatous skin show reduction in all appendages.

Cataract occurred in 48 cases, developing rapidly and bilaterally, before 7 years of age in 90% of cases, but sometimes as late as the third decade, as in our patient. Two siblings[28] had unusual eye changes remarkably similar to those of our patients, in particular atrophy of the anterior mesodermal layer and sphincter of the iris (Rieger's anomaly), a peripapillary crescent of chorioretinal atrophy, and tilted disc. Another patient had retinitis punctata albescens.[38]

Of the skeletal abnormalities, our patients have the saddle-nose reported in 14 previous cases, but not the severe shortening or absence of forearm or hand bones reported in 10 cases, or thumb hypoplasia described in 11 cases. Our patients have widespread bony abnormalities affecting joints, with subluxation of the head of the radius, humerus and femur, limiting joint movement. Limited elbow extension occurred in six other patients,[12,16,34,37,40,52] with an abnormal radial head in at least four, and two patients had subluxed knees.[33,40] There is little evidence that the joint contractures reported in RTS are due to soft-tissue contracture[52] rather than bony abnormality. The severe kyphoscoliosis in patient 1 also contributed to the diagnosis of Morquio syndrome[1] but has been reported in two patients with RTS.[31,40]

Metaphyseal chondrodysplasia, sclerosis and undertubulation of long bones are common in RTS[10,34,41,45,46,53] and the striking linear sclerosis affecting the femoral and tibial metaphyses illustrated by Kirkham and Werner[28] is similar to the changes in our patients.[2] This metaphyseal dysplasia may contribute to the characteristic short stature, and to the development of osteosarcoma (OS), which has now been reported in five patients with RTS: arising in the distal thigh of a 19-year-old boy,[15] the proximal tibial metaphysis of a 19-year-old boy,[31] the lower tibia of a 5-year-old girl,[46,62] the proximal tibia of a 13-year-old boy,[41] and the tibia of an 11-year-old boy.[53] A fibrosarcoma developed in the lower humerus of a 32-year-old man.[45] Two of these patients had pre-existing metaphyseal chondrodysplasia,[45,46] two had abnormal trabeculation of the long bones,[15,41] and of the two patients not previously X-rayed one had a sibling with RTS and metaphyseal dysplasia.[53] The association of OS with RTS has now been reported more often than would be expected by chance. Patients with RTS are considered cancer-prone,[45] and Smith *et al.*[63] found reduced DNA repair of hypoxic γ-irradiation damage in fibroblasts in two patients. However, there is no general increase in malignancy in RTS: gastric[30] and basal cell carcinoma[59] have occurred, but OS and squamous carcinoma of the skin are the only cancers reported more than once. Both tumours represent an occasional development in a tissue commonly dysplastic in RTS.

The extreme hypermobility of the fingers and wrists found in our patients previously suggested diagnoses of Morquio syndrome,[1] and connective tissue dysplasia.[2] However, similar joint hypermobility affecting the hands has been reported in brothers aged 2 and 1 with RTS and bluish sclerae.[17,18] The bone dysplasia mentioned above might also represent a collagen abnormality. Osteogenesis imperfecta has been diagnosed in two patients with RTS,[15,20] and a further four patients had multiple or pathological fractures,[34,37,45,54] which, like the joint hypermobility and the skin changes, have been predominantly acral in distribution. Fibroblasts from our patients produced normal types I, III and VI collagen, but clearly collagen studies should be pursued in this disorder.

Hypogonadism has been reported in 25 of the 59 patients aged 12 or over. In two, raised gonadotrophins suggested primary gonadal failure,[25,61] while in 10 low or normal gonadotrophins[18,25,28,29,48] or a small sella[27,57] suggest a primary pituitary defect. Pituitary dysfunction has also been suggested as an explanation for the resemblance between the dental abnormalities in RTS and those of panhypopituitarism and hypophysectomized laboratory animals.[32] Furthermore, in the 59 adult patients, saddle-nose occurred in 10 out of 25 hypogonadal patients but in none out of 34 endocrinologically normal patients: thus, 40% of patients who will remain sexually undeveloped can be recognized in childhood by the presence of saddle-nose. This is consistent with Kaufman's suggestion of a local maldevelopment affecting both mid-face and pituitary, based on an 11-year-old girl with RTS, growth hormone deficiency and saddle-nose.[56] The iris anomaly of Rieger, found in our patients and three others with saddle-nose and hypogonadotrophic hypogonadism[28,29] may have the same basis, being independently linked with mid-face hypoplasia (Rieger syndrome), growth hormone deficiency[64] and short stature (SHORT syndrome[65]).

Hypertension was reported in three girls with RTS[39,47] and in one,[47] renal biopsy showed calcified nodules in the endothelial cells of arteries. Renal biopsy was not done in our patient with hypertension but it seems likely that his hypertension results from chronic pyelonephritis unrelated to RTS.

Deafness due to middle-ear disease has been described in three other patients with RTS[11,53,58] but is probably common enough for this to be a chance association. A history of chronic infection in the ears, renal tract and skin prompted immunological investigations, but no abnormality was identified on routine testing. Haneke found impaired cell-mediated immunity in a patient with RTS[38] but there is nothing else to suggest an associated immune defect.

The pathogenesis of RTS is unknown. Inheritance is autosomal recessive[52] with an unexplained female predominance of 1·4:1. There is as yet no clue to the underlying genetic abnormality or its localisation.

Our patients were originally diagnosed as having Morquio syndrome on the basis of the spinal abnormality, lax skin and joints, and mucopolysacchariduria. The diagnosis was not confirmed biochemically by identifying the mucopolysaccharide as keratan sulphate, and was later excluded. The early finding of mucopolysacchariduria could have been erroneous as the method then used is difficult to quantify and there is little difference between the normal and abnormal ranges.[66,67] Furthermore, with urinary tract infection urinary mucoprotein can give a false positive result.[67] However, urinary mucopolysaccharide is elevated non-specifically in many degenerative disorders due to increased tissue turnover. Because the atrophic skin and juvenile cataracts of RTS suggest that this may be a degenerative disorder, Tokunaga[68] looked for, and found, raised mucopolysaccharide in a patient with RTS. We do not know which of these mechanisms explains the transient mucopolysacchariduria in our patients.

Over the years, the syndrome has become more clearly delineated in the literature. However, the cardinal feature remains the poikiloderma arising in infancy: ironically Poikiloderma of Thomson was the first clinical diagnosis recorded in the patients' case notes, before biochemical tests obscured the picture. The association of cutaneous telangiectasia and atrophy with Morquio syndrome has unfortunately become enshrined in the dermatological literature,[69] as a result of the earlier case report.

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Vesiculo-bullous Darier's disease

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SUMMARY

A 49-year-old man developed a widespread vesiculo-bullous form of Darier's disease following surgical drainage of a submandibular abscess.

CASE REPORT

A 49-year-old man with Darier's disease presented with a 2-day history of fever, dysphagia and a tender facial swelling. His only medication was etretinate (Tigason^R) 50 mg daily. Examination revealed a right submandibular abscess. He was treated with dexamethasone to reduce the swelling, intravenous antibiotics were given and the abscess was drained. Post-operatively his condition improved and dexamethasone therapy was discontinued after 24 h.

Erythema and swelling of the hands and forearms were noted 48 h post-operatively and by the following day a papular rash had developed on the trunk. The antibiotic therapy was stopped but the fever recurred and tense blisters containing golden-yellow fluid developed on the hands and wrists (Fig. 1). The signs were consistent with Darier's disease with secondary infection of lesions on the hands. Intravenous antibiotics were reintroduced. Over the next 48 h the patient became erythrodermic. Vesiculo-bullous lesions appeared on the previously normal skin of the trunk (Fig. 2) and the Nikolsky's sign was positive. There were no mucosal lesions. Maceration and friction led to the loss of sheets of epidermis. The patient became oedematous and increasingly unwell.

Investigations

Staphylococcus aureus was cultured from the submandibular abscess and from the blisters on the hands. No bacteria were isolated from blood cultures and no viruses or bacteria were isolated from the vesicles. The serum albumin was low, being 26 g/l.

The roof of an intact vesicle on the trunk was removed and frozen for rapid processing and this contained occasional dyskeratotic and acantholytic cells, but no basal cells were present. An excisional skin biopsy of vesicular lesions on the trunk confirmed that blistering was secondary to Darier's disease. There was suprabasal clefting with intraepidermal vesiculation, dyskeratosis and acantholysis (Fig. 3).

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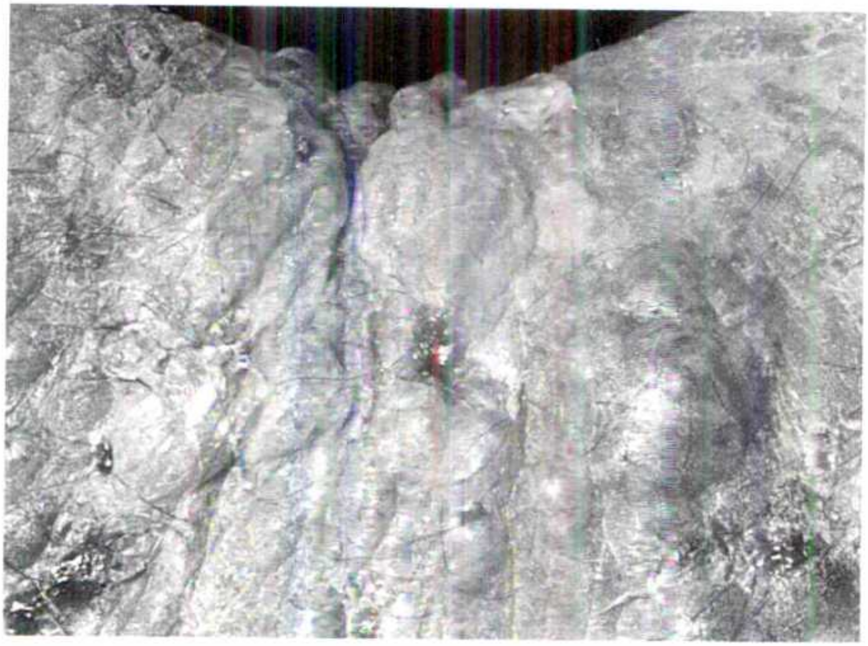

FIGURE 1. Tense blisters filled with golden fluid present on the hands and wrists. *Staphylococcus aureus* was cultured from the blister fluid.

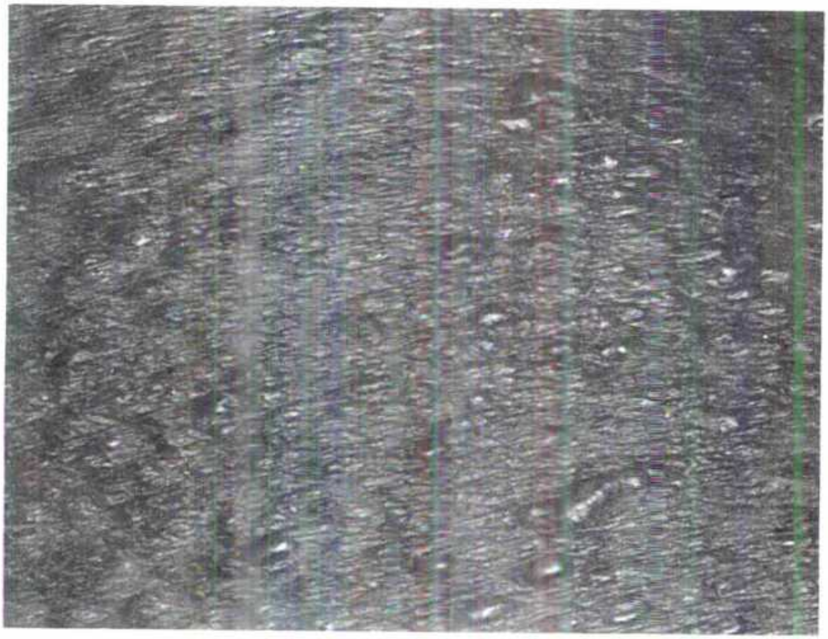

FIGURE 2. Vesiculo-bullous lesions and widespread erythema on the previously normal skin of the trunk.

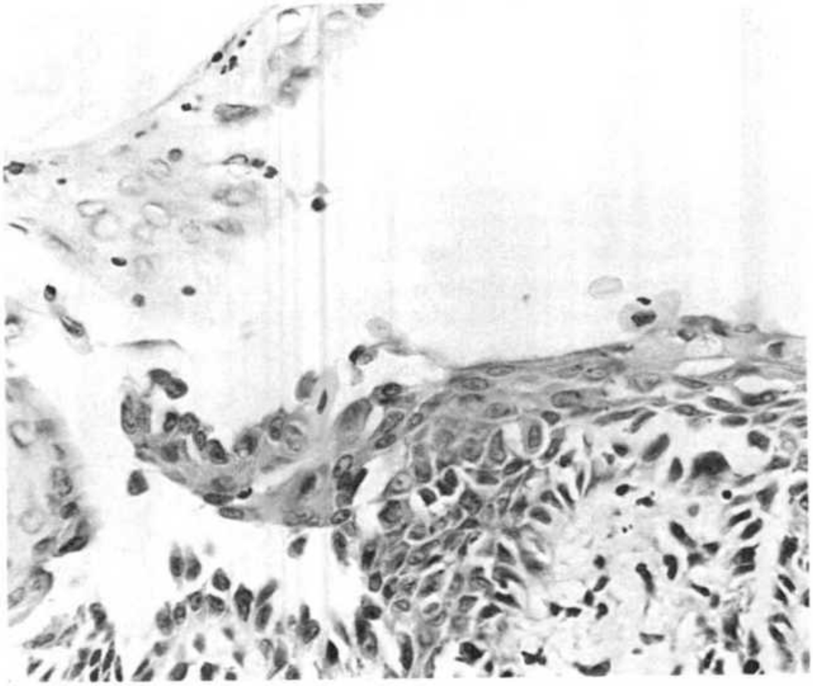

FIGURE 3. Biopsy from a vesicle. Vesiculation is secondary to Darier's disease. There is dyskeratosis, acantholysis and suprabasal clefting forming an intraepidermal vesicle. (Haematoxylin and eosin, × 400.)

Management

The care of the large areas of eroded skin became increasingly difficult and the patient was transferred to the Intensive Care Unit where he was nursed on a Clinitron bed (Systems Support Int., Medical Services Ltd, Finch Close, Nottingham, U.K.). The eroded skin was dressed with 10% povidone iodine ointment under paraffin tulle gras. The hypoproteinaemia was treated with intravenous albumin with the resolution of the oedema and treatment with flucloxacillin 500 mg q.d.s., gentamicin 80 mg t.d.s. and etretinate 50 mg daily was continued.

Following a febrile episode associated with profuse sweating more skin was lost from the shoulders and back. Clobetasol propionate 0·05% ointment was applied to the inflamed and eroded areas. The patient gradually recovered and was discharged from hospital 4 weeks after admission.

DISCUSSION

Darier's disease is a dominantly inherited disorder of keratinization. Typically the disease presents around puberty with hyperkeratotic papules, which may coalesce to form verrucous areas especially involving the flexures. The histological features include suprabasal clefting, lacuna formation, acantholysis and dyskeratosis.[1] Darier's disease has similar clinicopathological features to Hailey–Hailey disease (benign familial pemphigus), with which it may be confused.[2,3] Although there are descriptions of the two diseases occurring simultaneously, [2,3] it is possible that many of these cases had bullous Darier's disease. Family studies and histological observations suggest that the diseases are separate entities.[4] Discrete vesicular lesions, caused by enlargement of the intraepidermal lacunae, may occur in Darier's disease and blistering may be precipitated by factors such as a high humidity, ultraviolet radiation, surgery or physical stress.[4,5] Widespread vesiculation or bullous lesions are unusual in the absence of cutaneous bacterial or viral infections. The combination of systemic bacterial infection, surgery, pyrexia, sweating and stress may have triggered the blistering seen in our case.

The differential diagnosis of such rapidly progressive vesiculation in Darier's disease includes cutaneous viral infection, both herpes simplex and herpes zoster, bacterial infection, the staphylococcal scalded skin syndrome (SSSS) and drug-induced toxic epidermal necrolysis (TEN). A skin biopsy is essential but an early diagnosis can be established by the examination of a frozen section of a blister roof. It is possible to differentiate TEN, in which the roof is composed of a necrotic full-thickness epidermis and SSSS, where the split is through or just below the granular layer, from Darier's disease. Bacterial and viral cultures must be performed or viruses may be detected by electron microscopy.

Paradoxically etretinate, the retinoid used to treat Darier's disease, may in our patient have predisposed him to the widespread cutaneous loss. *In vitro* studies show that the retinoids inhibit desmosome production and interfere with cell adhesion[6] and *in vivo* systemic retinoids induce desquamation and skin fragility.[7] Although patients taking oral retinoids rarely develop blisters, cutaneous erosions are common in those treated with etretinate.[8] Studies have demonstrated attenuation and loosening of the stratum corneum as well as loss of desmosomes.[9] In our patient, etretinate may have contributed to the formation of bullous lesions and the skin fragility.

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British Journal of Dermatology (1990) **122,** 835–836.

Obituary

Professor George Findlay

George Findlay passed away in November 1989. For many of us who knew him well, this marked the end of an era in which he dominated dermatology in South Africa, established an international reputation as an original thinker and researcher, and pursued a variety of interests with unflagging enthusiasm and skill. Even so, he spent much good time with his beloved family, and never neglected his friends and those for whom he cared. With so much to do, he was protective of his time, and wasted very little of it. He endured his long illness with optimism and courage, and only abandoned his medical practice a month or so before he died. He never allowed his many interests to interfere with his work, so his lifestyle meant business as usual until the end.

George was an extraordinarily interesting man. His inquiring mind and thirst for knowledge brought him four distinctions when he matriculated at Pretoria Boys High in 1939. He graduated in medicine with honours at the University of Witwatersrand in Johannesburg in 1946, having acquired a B.Sc. Honours in Zoology on the way. He specialized in dermatology under James Marshall, and gained his M.D. degree by thesis from the University of Pretoria in 1951. He then went overseas to gain further experience. An Eli Lilly Fellowship brought him to the University of Chicago in 1953. Here he was involved in Stephen Rothman's final preparations for his textbook on physiology of the skin, and also worked in dermatopathology with George Gomori, for whom he had a lasting admiration. He went to Duke University to acquire a certificate in medical mycology under the direction of Norman Conant during his stay in the U.S.A. Britain and Europe were to become happy hunting grounds for George, and his dermatological travels were enhanced by his ability to speak French, German, and Dutch.

He returned to Pretoria in the mid-1950s to pursue a career as academic teacher, researcher, and clinical dermatologist, which was to last 35 years. He had already published scientific papers as a medical student and later as a registrar, but the flow of publications was to intensify and continue. His final tally was three books, 15 book chapters, and some 150 scientific articles, not to mention review articles, editorials, and book reviews. The wide range of subjects he wrote about reflected his interest in zoology, botany, anthropology, and palaeontology, in addition to dermatology. His publications led to the award of the D.Sc. degree by the University of Witwatersrand, his Alma Mater, in 1961. George was appointed Head of the Section of Dermatology at the University of Pretoria in 1956, when he was 32 years old, and he held that post until his retirement in 1988. In 1966 he was appointed to a personal chair, the first professorial appointment in dermatology in South Africa, a signal honour for a university part-timer in those days. During his tenure, he fully trained 13 dermatologists, and four others completed part of their registrarships with him.

His researches led him to a lasting association with the Council of Scientific and Industrial Research when he became Director of its Photobiology Research Group when it was founded in 1959. This association lasted until 1989, when the group became incorporated into the Research Institute for Environmental Disease. George spent his mornings there in research, following his retirement from the university. His expertise in photobiology brought him international recognition, and he served as Vice-President of the 'Comité International de Photobiologie' from 1964–8. His researches in other fields, and his ability as a lecturer, added to his reputation. He delivered the Prosser White Oration at the Royal College of Physicians in 1967. He was visiting professor at the University of London in 1970 and 1972. He was elected to honorary life membership of the South African, British, American, French, Netherlands, and Israeli Dermatological Societies. He became a life member of the Royal Society of South Africa, the Royal Society of Medicine, and the American Society for Investigative Dermatology. His untimely death prevented him from lecturing in Belgium and Edinburgh in late 1989, and from delivering the Centenary Dowling Oration in London in 1991.

With all of this, George was a superb clinician. He learned a lot from others, both in South Africa and overseas, but a lot of what he knew was self-taught, based on his own reading, observations, and analytical skills. He was always ready to exchange observations and ideas with his staff, and with colleagues, notably

L.J.A. (Alfonse) Loewenthal in the early days, and later on his close friend, F.P. (Fred) Scott. Clinical dermatology was an essential part of his entire professional life. His large private practice and his many hospital clinics and ward-rounds provided him with a unique clinical experience and perspective. He was a consultant's consultant and his opinion was sought by many with apparently insoluble dermatological problems, and few were disappointed.

George set himself high professional standards and rigidly adhered to them. He expected those who worked with him to do the same. A dermatological training with George Findlay was not to be undertaken lightly. He expected a total commitment from a trainee, and he did not suffer fools gladly. This was overpowering for some, but for others who displayed enthusiasm, initiative, and a desire to learn, it opened the door to an educational experience of extraordinary depth. Fortunately, his iron will and determination for excellence were tempered by his impish sense of humour, which could turn so many activities into fun. He had a wide appreciation of classical music, literature, art, good wine, and good company, and was a pianist of no mean repute. He was an excellent raconteur and his reminiscences were apt and rarely dull. George's enthusiasm was contagious, and his inquiring mind stimulated those around him to use theirs. His was a powerful influence, and for some, I know, it brought out the best in them, and was a turning point in their professional lives. George himself owed a great deal to two people who had a profound influence on him. The first was Robert Broom, a physician and famous palaeontologist, whom George got to know and admire in his student days. He was impressed by a man who made his living from medical practice but spent his spare time in the scientific research that he loved. The second was G.B. Dowling, that master of clinical dermatology, who was to become role model, friend, and 'Uncle Geoffrey' to George. Others influenced him, but these two helped to shape his life.

George's main support for his last 32 years was his wife, Paula, a constant source of love and serenity. Their five children and two grandchildren provided the background of affection and encouragement which allowed him to pursue his varied interests. His children chose not to compete with him and are happily engaged in different professions. It is clear, however, that the family tradition of original thought will continue, as he would have wished. Let us all remember George Findlay—there are not many like him in this life.

D.A. WHITING

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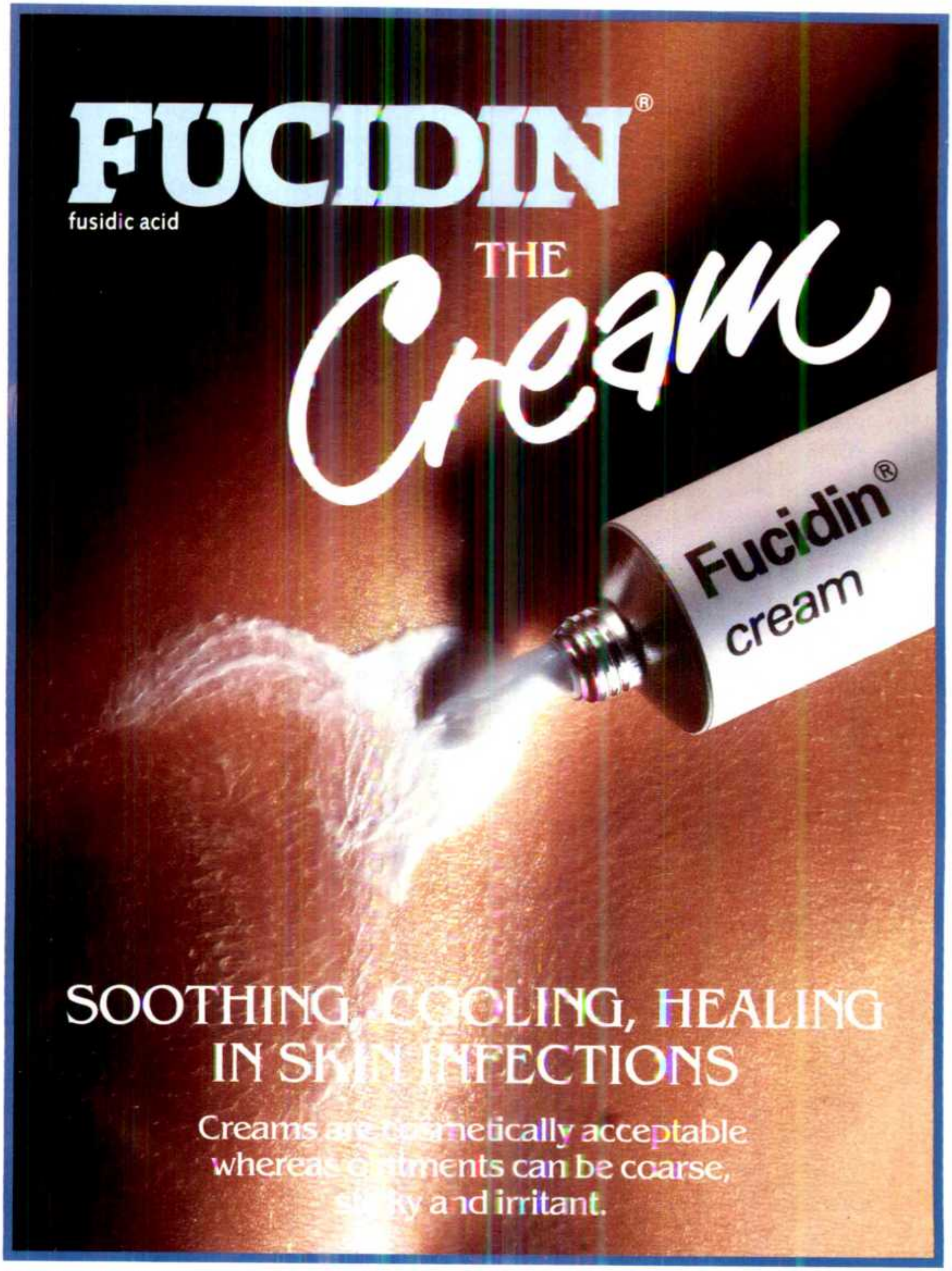
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L E O

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Requiem for toxic epidermal necrolysis

SIR, Toxic epidermal necrolysis (TEN)[1] has come to the end of its useful life and should be abandoned, because what I thought was a single entity of 'scalded skin' has turned out to be three separate diseases.

Of the four cases studied (see ref. 1), one (case 4) was a severe drug eruption, with features of erythema multiforme, attributed to phenylbutazone. Another (case 3) was an example of what came to be called the staphylococcal scalded skin syndrome. The other two did not fit these diagnoses. They gave no drug history, their uniform eruptions were unlike erythema multiforme, and both had repeated attacks.

Their probable diagnosis came to me through Kirsti Kauppinen's bold challenge experiment.[2] She gave substantial doses of the suspected drug to patients who had recovered from severe muco-cutaneous bullous drug eruptions. Patients with 'Lyell's syndrome' and 'Stevens–Johnson syndrome' behaved completely differently from those with generalized bullous fixed drug eruption (GBFDE). The former could sometimes be provoked, but only within the first 4 months. The reactions that occurred were usually mild, and never as severe as the original eruptions. In contrast, all patients with GBFDE reacted fully, and without time limit. There can be little doubt that cases 1 and 2 had GBFDE, because of their uniform eruptions and recurrences. The negative drug histories are not significant since it is notorious that the causative drug can be difficult to identify in fixed eruptions. Case 1 probably knew of and exploited a drug sensitivity.

A name other than the already compromised TEN, Stevens–Johnson syndrome or erythema multiforme major is necessary to describe patients such as in case 4. I suggest 'exanthematic necrolysis', which conveys the sudden outbreak of a widespread eruption and the prostration, and also provides a restrained echo of its now discredited parent, TEN. I prefer exanthematic necrolysis to Ruiz–Maldonado's 'acute disseminated epidermal necrosis'[3] because it is briefer and crisper, and also because the GBFDE has been excluded. However, I agree with him that it is a waste of effort to try and distinguish between what were known as TEN and the Stevens–Johnson syndrome, since they represent aspects of the same spectrum of reaction.

While there are clinical and probably histopathological differences between exanthematic necrolysis and GBFDE (for example more severe mucosal lesions, a multiform eruption and higher mortality in the former), they are not so definite as to make the distinction possible in every case. However, using modern techniques to unravel the mechanism of the fixed eruption, the distinction between them could be determined. It is almost certain that the mechanisms differ: a temporary state exists in exanthematic necrolysis during which a drug can provoke the reaction (not necessarily by an immune mechanism), whereas it seems as if in GBFDE parts of the skin become, and remain, truly sensitized to a drug. Reactive areas could be compared with non-reactive ones, and there is an animal model available.[4] Although there is no model for exanthematic necrolysis, investigation of the co-factor or co-factors (which are believed to include upper respiratory tract infections) would be possible.

An important corollary of this view of exanthematic necrolysis is that suspected drugs should be avoided in acute infections—specifically, that non-steroidal anti-inflammatory drugs (NSAIDs) must not be used to combat the febrile symptoms of 'influenza'. The need for a co-factor could explain the notably low incidence of exanthematic necrolysis compared with the heavy usage of the suspected drugs. The rare and serious Reye's syndrome, believed to be caused by giving the commonly prescribed drug aspirin to children with acute viral infections, is analagous. The hypothesis that Reye's syndrome is due to augmented release of tumour necrosis factor from macrophages under the influence of NSAIDs,[5] could have a bearing on the mechanism of exanthematic necrolysis.

The demise of TEN need occasion no regret; but perhaps it might be noted in passing that its existence contributed to our knowledge of staphylococcal epidermolysins, that it engendered the concept of exanthematic necrolysis, and that it points to the fixed eruption as a worthwhile subject for research.

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Neuropeptide-like immunoreactivity in skin lesions of atopic dermatitis and psoriasis

SIR, Several neuropeptides (NP) have been found in nerve fibres of normal human skin using immunohistochemical as well as radioimmunological techniques.[1] In peripheral tissues they are thought to be the main mediators of neurogenic inflammation, which can be involved in the pathogenesis of some inflammatory dermatoses.[2] We would like to report our observations on the immunohistochemical pattern of several NP immunoreactivities in the lesions of atopic dermatitis (AD) and psoriasis.

Skin biopsies were taken from lesional lichenified skin (from the flexor surface of the elbows) of 11 patients with AD and from lesional skin of 10 patients with chronic plaque psoriasis. Biopsies were also taken, as controls, from the same areas of six healthy volunteers, from the uninvolved skin of eight patients with psoriasis and from two subjects with prurigo lesions. Frozen sections were processed using an indirect immunofluorescence method (biotin–streptavidin system) to evaluate the distribution pattern of eight NP (substance P, calcitonin gene-related peptide, neurotensin, vasoactive intestinal polypeptide, neurokinin A, galanin, neuropeptide Y, somatostatin).

We found that substance P-like immunoreactivity was intensely expressed around the vessels in the superficial dermis of AD patients, but not in the normal controls. The number of somatostatin immunoreactive free nerve endings in the dermis was slightly increased in controls as compared to involved atopic skin. NPY-like immunoreactivity was detected within dendritic epidermal cells in six out of 11 patients with AD. There was no difference in the occurrence of the remaining NP in atopic and healthy subjects. These results seem to confirm our previous data concerning a possible role of NP in the pathogenesis of AD, which was indicated by the altered skin reactivity to intradermally injected NP in AD patients.[3]

In psoriatic patients the immunoreactivity pattern of the NP in lesional skin did not significantly differ from that in lesion-free skin nor from that in healthy controls. These data are in agreement with those reported by others.[4,5] Naukkarinen *et al.*[6] using morphometric quantitative techniques have recently shown an increase of substance P-containing nerves in the epidermis of lesions of psoriasis. Further studies will be necessary to evaluate the role for peptidergic mediators in these and other inflammatory dermatoses.

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Fluconazole in the treatment of refractory oral candidiasis and tinea pedis in an elderly patient

SIR, A case is reported of fungal infections of the mouth and skin which, having proved resistant to all available topical and systemic antifungal agents, responded to the new antifungal drug fluconazole.

A 79-year-old woman with hypothyroidism and rheumatoid arthritis presented with a fungal infection of the left foot and calf. Her main complaint was of a severe burning sensation of the tongue which restricted her to having a bland diet for over 2 years. Culture of the furred tongue produced an abundant growth of *Candida albicans*, and culture of the lesions on the leg yielded *Trichophyton rubrum*, with a mean inhibitory concentration of 1·0 μg/ml for griseofulvin and 2·0 μg/ml for ketoconazole. Over the next 11 months her leg and foot were treated topically in turn with clotrimazole, miconazole, econazole, Castellani's paint and bifonazole. For the first 4 months micronized griseofulvin, 0·5 g was given daily and for the following 2 months, ketoconazole 200 mg daily in conjunction with the topical treatment. The only drug that produced a slight improvement of the symptoms was bifonazole. The oral candidiasis was treated with amphotericin B lozenges, 10 mg four times daily, for periods of 2 weeks at a time and this, to some extent, relieved the burning sensation of the tongue. We then obtained a supply of fluconazole, a new bis-triazole agent, with the approval of the Norwegian National Board of Health and this was given orally in a dose of 100 mg/day for 8 weeks followed by 50 mg/day for a further 8 weeks. After 4 weeks, all her symptoms and signs had cleared, and after 7 weeks the symptoms of her oral candidiasis had also disappeared.

Three weeks after discontinuing fluconazole the candidiasis relapsed and amphotericin B lozenges were again administered for 3 weeks, but without relieving the burning sensation of the tongue. Fluconazole was reintroduced at a dose of 50 mg/day for 4 weeks and the patient became symptom free before the end of the course. Fluconazole maintenance therapy was then initiated at a dose of 150 mg once a week and has since been continued for 23 months without any further relapse of the fungal infection.

Skin scrapings from the calf and foot became negative for fungi on microscopy and culture during the first 2 months of treatment with oral fluconazole. The oral cavity has however remained culture positive for *C. albicans*, although the oral load of the fungus has been markedly reduced and the patient is symptom free. Regular routine haematology and tests of hepatic and renal function have revealed no abnormalities. We propose to continue indefinitely with prophylactic oral fluconazole, 150 mg once weekly, since this antifungal agent appears to be effective and well-tolerated.

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A transient bullous eruption associated with adenovirus infection in a child

SIR, We wish to report a child who, in 1976, developed a transient bullous eruption associated with adenovirus infection. Recent advances in virology suggest that this case may now have more general significance.

In mid-March 1976, a 4-year-old boy was seen with blistering of the skin on his back, genitalia and legs and with a few lesions on his shoulders and ears. The blisters varied in size from several millimetres up to

1 cm and contained clear fluid. There were no mucosal lesions and the child was otherwise healthy. New lesions developed in groups on erythematous skin and in crops over succeeding days. No systemic treatment was given and the condition resolved after 3 months. There has been no recurrence to date. One month before admission he had been unwell with headache, nausea and abdominal pain but no bowel upset. He had received at that time a 10-day course of co-trimoxazole. His full blood count and the serum immunoglobulins were normal. The blister fluid was examined by electron microscopy for viral particles with a negative result and there was no evidence of bacteria in the blister fluid either by Gram's stain or on culture. The histopathology was of a subepidermal bulla containing fibrin and with numerous neutrophils and a few eosinophils. Direct immunofluorescence of the biopsy was negative for immunoglobulins but there was a patchy distribution of complement and fibrin at the basement membrane. The complement fixation titre for adenovirus on 1 April 1976 was 1:16 rising to 1:256 by 28 April 1976.

It has been recognized that patients with bullous disorders, including that of chronic bullous disease of childhood, may have a prodromal illness[1] and an infective factor has been suggested.[2] Chronic bullous dermatosis of childhood is characterized by the presence of linear IgA deposits at the dermo-epidermal junction[3] and there are a number of other distinctive bullous disorders occurring in children.[4] Adenovirus infections are common and self limiting.[5] In recent years adenovirus has been implicated in the pathogenesis of coeliac disease[6] and a sequence homology between A-gliadin and a structural protein of adenovirus has been reported.[7] It would be of interest to know if other centres have found an association between the occurrence of adenovirus infection and the development of chronic blistering dermatoses in children.

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Deliberate self-poisoning with isotretinoin

SIR, Isotretinoin is now a well-established treatment for severe cystic and conglobate acne which has failed to respond to other therapies. To date there are only two recorded cases of acute isotretinoin poisoning in the literature.[1,2] In neither case were significant clinical or laboratory abnormalities detected; but the patients did not present until 2 and 5 days following ingestion of isotretinoin.

I report a further case of deliberate self-poisoning with isotretinoin by a 15-year-old, 54 kg, female patient. She had severe acne which had failed to respond to either erythromycin or cyproterone acetate.

Isotretinoin was started at a dose of 50 mg/day after baseline tests for liver function and fasting serum lipids had been carried out. Two months later she ingested a total of 350 mg isotretinoin following an argument at home and presented to the casualty department some 1.5 h later where gastric lavage was performed.

She had attended the clinic for routine follow-up earlier the same day and the results of the liver function tests and fasting serum lipids were available for comparison with those obtained following her self-poisoning with isotretinoin. All these had been normal and over the following 4 weeks her full blood count, liver function tests, clotting screen and fasting serum lipids showed no significant abnormalities. She was followed-up for a total of 5 months and her only complaint was that of vague abdominal discomfort 48 h after the ingestion which resolved spontaneously. In particular she had no headache or nausea and there was no evidence of papilloedema, hepatic tenderness or dry skin. She had no further treatment with isotretinoin.

It was not possible to determine the amount of isotretinoin absorbed into the circulation in this case because the serum must be protected from light and stored at −20°C prior to assay. Serum taken 7 and 20 days following the ingestion was analysed by F.Hoffman–La Roche and Co. Ltd, Basle, and showed only insignificant levels of isotretinoin but 5 ng/ml and 2 ng/ml respectively of Ro22-6595, the 4-oxo metabolite. The average half-life of isotretinoin in the circulation is 23 h therefore levels of this order would be consistent with a significant overdose. However, levels of this order have been detected in patients not taking oral retinoids, as a consequence of endogenous retinoic acid metabolism (Dr C.Brindley and Dr R.Green, personal communication).

As isotretinoin treatment becomes more common the number of episodes of self-poisoning will inevitably increase. It is, therefore, reassuring that no significant abnormalities have been detected in this or the other two reported cases.

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Lichen planus-like eruption and nail changes in a patient with graft-versus-host disease

SIR, Nail changes as a manifestation of graft-versus-host disease (GVHD) following bone-marrow transplants are identical in some cases to those seen in typical lichen planus with fluting and onychatrophy.[1] We describe a man who developed scarring nail changes with skin and mouth involvement in association with chronic GVHD.

A 40-year-old man presented with mouth ulcers, lethargy and generalized aches in 1986 and was diagnosed as having a myelodysplastic disorder. Six months later, June 1987, he developed acute myeloid leukaemia, which was treated with chemotherapy with remission. He was later given an HLA-matched allogeneic bone-marrow transplant from his brother and 1 week afterwards the patient developed a mild, scaly, maculopapular rash on his trunk and mouth ulceration, and acute graft-versus-host disease was diagnosed. He was treated with prednisolone and cyclosporin. Two months after the transplant, in November 1987, his rash and mouth ulceration had cleared, but at this time he developed a pneumonitis and mildly abnormal liver function tests that subsequently improved. Cyclosporin was continued until February 1988 and prednisolone until June 1988. In August, his skin rash reappeared and prednisolone was restarted. Thalidomide was added in October as the rash had persisted. A skin biopsy taken from his trunk at this time showed vacuolar degeneration of the basal cell layer of the epidermis, pigmentary incontinence and a mild dermal lymphocytic infiltrate with a perivascular tendency and a few eosinophilic bodies. These changes were characteristic of the lichen planus-like eruption in chronic GVHD (Fig. 1).

In November 1988, his finger-nails started to grow abnormally and gradually became dystrophic (Fig. 2) and by January 1989 his toe-nails became similarly affected. There appeared to be destruction of the nail matrices. At this time there were poikilodermatous changes with pigmentation and sclerosis of the skin on the trunk. The histology of a nail matrix was non-specific and showed an atrophic epidermis with an increased amount of dermal collagen.

GVHD complicates about 70% of allogeneic bone-marrow transplants despite the use of HLA-matched sibling donors, and the administration of immunosuppressive agents.[2] The main targets of GVHD are the skin, the gastrointestinal tract and the liver. Skin involvement is present in all patients with GVHD. Acute GVHD develops within 2–3 months with a maculopapular rash, diarrhoea and liver involvement. Chronic GVHD develops in 10% of all patients undergoing allogeneic bone-marrow transplants[2] and may develop *de novo*, a gradual progression from acute GVHD, or following resolution of acute GVHD as in our patient.

Saurat *et al.*[3] described 10 cases of lichen planus-like eruption following 34 bone-marrow transplantations. It only occurred after the persistent chronic phase of GVHD and in all cases there was mouth involvement. A particularly disabling type of chronic generalized GVHD, demonstrated in our patient, is the late phase which develops after a year and in which there is severe poikiloderma with widespread cutaneous sclerosis.

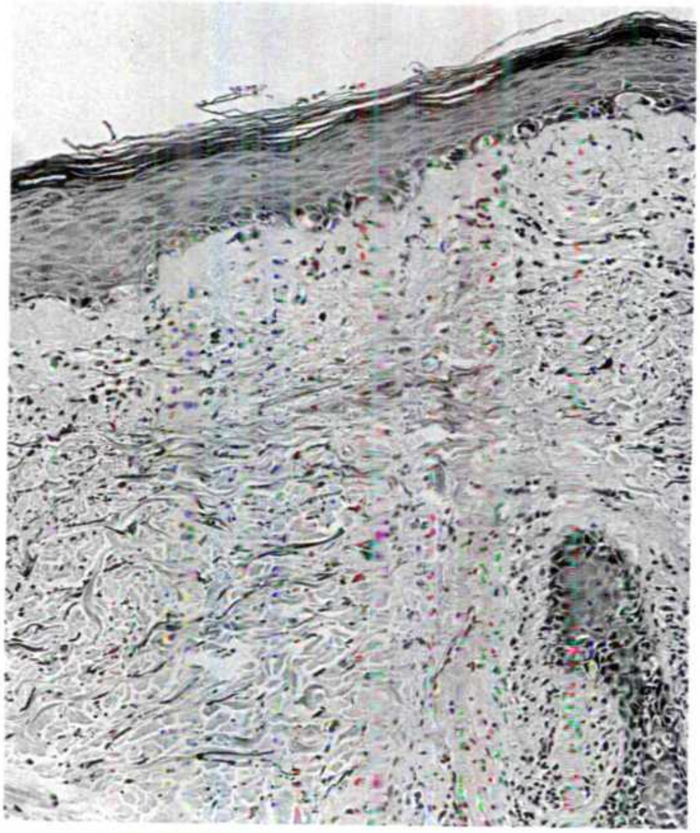

FIGURE 1. Skin biopsy showing basal liquefaction and perivascular chronic inflammatory cell infiltrate.

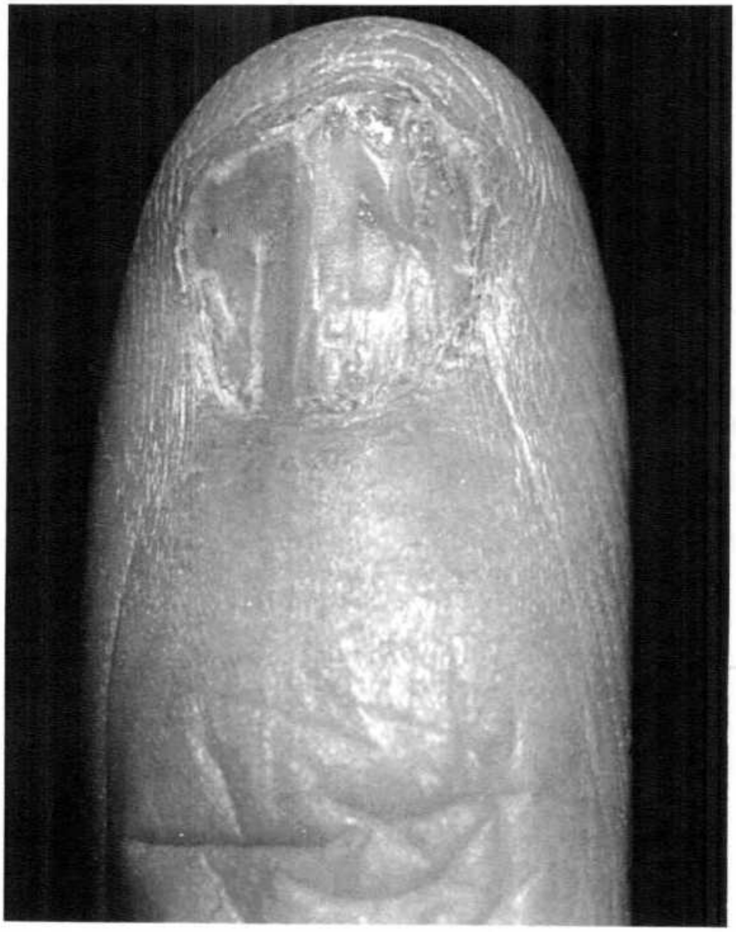

FIGURE 2. Close up of finger-nail.

The similarity in pathogenesis of idiopathic lichen planus to GVHD is further supported by the previously unreported finding of pterygium developing in our patient with chronic GVHD.

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Agminate lichen follicularis with cysts and comedones

SIR, Retroauricular follicular lichen planus tumidus (FLPT)[1] and milia en plaque[2] are unusual entities of uncertain nosography. We describe a patient with the clinical features of both conditions. The direct immunofluorescence and immunohistochemical findings were typical for lichen planus pilaris (LPP).

A 57-year-old woman presented with asymptomatic erythematous and oedematous plaques in both retroauricular folds and milium-like whitish papules that developed several months previously (Fig. 1). Two similar lesions were present on her chin and right cheek. In both conchae there were innumerable comedone-like lesions. The scalp and body hair, nails and mucous membranes were normal. She denied taking any drug.

Laboratory investigations were either negative or normal, including antinuclear and anti-ENA antibodies. Histopathology showed that in the dermis there were large follicular cysts filled with orthokeratotic material and remnants of hair follicles, surrounded by a dense lichenoid band-like lymphocytic infiltrate. Some colloid bodies were also seen. Alcian blue stain did not reveal deposits of mucin in the hair follicles. Direct immunofluorescence showed granular deposits of C3 at the dermo-epidermal junction, and IgA, IgM, C3 and C4 coated bodies in the upper dermis as well as linear deposits of fibrinogen around the adnexa and blood vessels and C3 deposits in the dermal vessel walls. The vast majority of the mononuclear cells in the dermal infiltrate were T (CD3$^+$/CD5$^+$) lymphocytes, most of them being of the helper/inducer (CD4$^+$) phenotype. Cytotoxic/suppressor (CD8$^+$) cells were much fewer and at the periphery of the infiltrate. The CD4/CD8 ratio was 3:1. Large numbers of Langerhans (CD1a$^+$) cells were seen in the epidermis. Some lymphocytes and epidermal keratinocytes were HLA-DR$^+$.

Isotretinoin was begun at a dose of 40 mg/day and in 4 weeks the cysts and comedones flattened and reduced in size. The dose of isotretinoin was reduced to 20 mg/day but the lesions recurred with intense

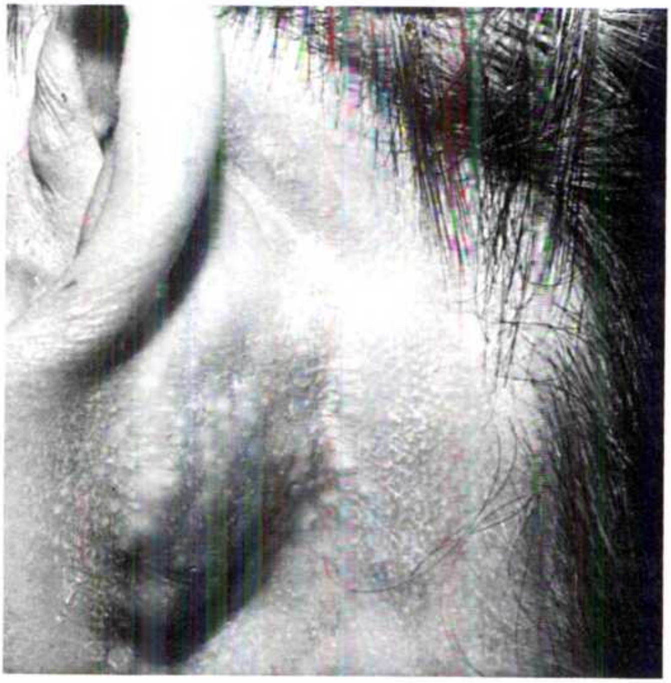

FIGURE 1. Retroauricular erythematous and oedematous plaque with milium-like cysts.

itching. Prednisone (0·5 mg/kg per day) was then given and the patient improved, but any attempt to reduce the dose was followed by relapse. Six months later a plaque of alopecia developed on the vertex of her scalp.

Follicular lichen planus tumidus (FLPT) has been described in eight patients.[1,3] The male/female ratio is 1 : 2 and the age range is 20–63 years (average age 42·5). Clinically there are reddish/violaceous plaques with innumerable whitish cysts and comedone-like lesions in the retro-auricular areas. Bilateral FLPT has been reported in only two cases, including our patient. Only exceptionally is this area spared. Other areas that may be affected are the neck, cheeks, chin, ears and scalp (our case only). The tumid features were present in five of the cases. Itching is an occasional symptom. Four patients have been reported as also having typical lichen planus (LP) lesions and a further one had lichen sclerosus et atrophicus. In all the cases, the histopathology was that seen in LPP. Our case is unique in that she developed alopecia. The immunopathological findings in our case were that of early LP.[4]

Other diseases that were considered in the differential diagnosis included discoid lupus erythematosus but this was ruled out by the direct immunofluorescence and immunohistochemical studies. Nodular elastosis with cysts and comedones (Favre–Racouchot syndrome) could be differentiated in that it occurs in the sun-exposed areas and mainly in the periorbital region in elderly people. The histopathology in this condition is dominated by severe elastosis with an absence of any lichenoid infiltrate. Follicular mucinosis was excluded by the lack of mucin in the hair follicles. Milia en plaque[2] defines the only two cases in the English literature that could be FLPT, differing only in their less apparent lichenoid features.

Treatment in this disorder is disappointing and topical as well as systemic steroids only improve the lesions. Topical tretinoin is ineffective. In our patient, treatment with isotretinoin initially improved the condition with clearing of the lesions, but was not tolerated and attempts at reducing the dose failed. Only in one of the reported cases did lesions clear spontaneously after 44 months.

In conclusion, LPP may uncommonly present with milia and comedones on an inflamed plaque and this is best termed agminate lichen follicularis with cysts and comedones.

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The French Triple Centenary, 12 October 1989

Sir, We were fortunate enough to be invited to attend the combined centenary of the foundation of the Société Francaise de Dermatologie et de Syphiligraphie, the first International Congress of Dermatology and the inauguration of the Museum of the Hôpital Saint-Louis. This unique celebration was held in Paris on 12 October 1989. The afternoon session of the Society consisted of an ingenious and entertaining reconstruction of the first meeting held in August 1889. Under the Presidency of P.Amblard and Madame Sayag, each of the four main topics were paired with similar ones given then but, on this occasion, preceded by an historical resume of the Society's subsequent 100 years. Lichen planus and the lichenoid eruptions were elegantly presented by Grosshans, as were the congenital erythrodermas by Larrègue. Saurat reviewed the acquired bullous eruptions and Madame Sayag progress in the trichophyton infections. The session ended with an evocative wide-ranging socio-philosophical review of venereology by Janier.

The social events began with an enchanting concert of seventeenth and eighteenth century music played on flûtes à bec, viole de gambe and clavecin in the chapel of the St Louis Hospital (founded by Henri IV in 1607) and ended with a splendid banquet in the museum, surrounded by Baretta's remarkable moulages; a most impressive setting.

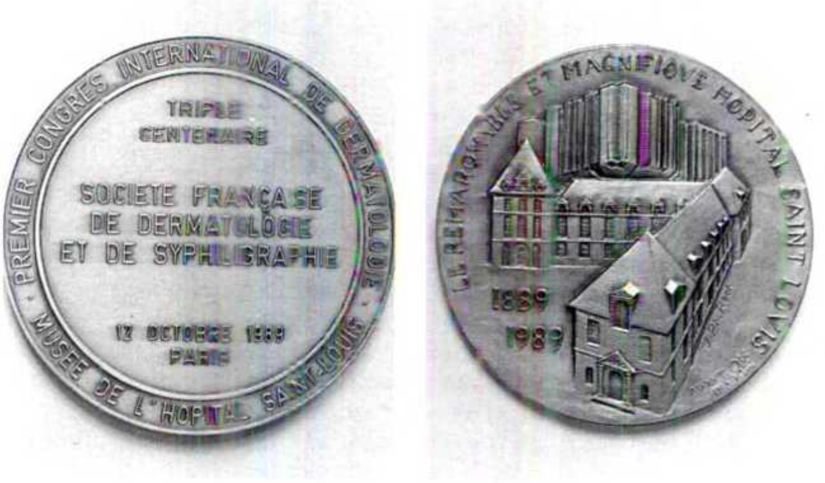
PREMIER CONGRES INTERNATIONAL DE DERMATOLOGIE
MUSEE DE L'HOPITAL SAINT LOUIS
TRIPLE CENTENAIRE
SOCIETE FRANÇAISE DE DERMATOLOGIE ET DE SYPHILIGRAPHIE
12 OCTOBRE 1989 PARIS
LE REMARQVABLE ET MAGNIFIQVE HOPITAL SAINT LOVIS
1889
1989

FIGURE 1. Commemorative medal by J.Ph.Roch.

The participants in this réunion came away, not only with a handsome medallion by J.Ph.Roch, struck for the occasion (Fig. 1) and a delightful print of the St Louis Hospital but with the lasting memories of a very special occasion, marked with elegance and style. We ourselves were honoured to have been invited and to have been able to pass on the warmest greetings of the B.A.D. to our friends 'across the water'.

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Book Reviews

Pathology of the Skin: with Clinical Correlations. P.H.McKEE (1989). London: Gower Medical Publishing. Pp. 558. Illustrations: 2238. ISBN 0-397-44601-2. Price £120.00.

This is a beautiful book, lavishly illustrated in colour with photo-micrographs, correlated wherever possible with clinical pictures, and it is these features which will make this volume an excellent buy for the general pathologist at whom the book is aimed.

There are many points which will endear the book to the dermatologists who have an interest in dermatopathology, not least being the illustrations of the varying histological appearances sometimes seen in the same skin lesion. They will also appreciate the chapter on Cutaneous Metastases. The chapter headings are the ones to be expected and at the end of each chapter there are up-to-date references, (the latest found being a paper from 1987), but older important papers are also listed.

The text is somewhat terse with an economy of words which is often barely adequate, and can be awkward, especially with regard to definitions and explanations. Occasionally it becomes misleading, for instance only 3% of patients with chronic discoid lupus erythematosus have oral lesions—not the 25% referred to in the text which really relates to systemic lupus erythematosus, and there are other errors of this kind. As with many other 'first' texts of books there are numerous spelling mistakes including the common 'pruritis' to be found, together with at least one other awkward neologism 'neurotization'.

A short glossary of the somewhat esoteric terms used in dermatology might also have been included to help the many general pathologists who buy the book. The inclusion of many electron microscopy photographs is very helpful in the absence of a current comprehensive atlas on electron microscopy changes in diseases of the skin.

The pages are numbered by chapter rather than sequentially through the book, and the illustrations are numbered in the same way. This is sometimes slightly confusing, but the index is adequate and the text is usually close to the relevant picture.

The standard dermatopathology reference book to which all others have to be compared is Lever's '*Histopathology of the Skin*'. Whilst by no means replacing Lever, McKee stands up well to the comparison, even taking into account the recently published atlas to accompany the Lever book. The price is high but the production is excellent and the histopathology atlas with clinical correlations make the book worth buying on this account alone. Despite being a physically large book and awkward to hold, it is a volume which can be readily recommended to both dermatologists and general pathologists.

T.W.STEWART

Radiation Measurement in Photobiology. Edited by B.L.DIFFEY (1989). London: Academic Press. ISBN 0-12-215840-7.

This is a book for those active in the field of photobiology, including dermatologists, photochemists, physicists, zoologists and botanists. The book outlines some of the fundamentals and also describes some actual measurement techniques with reference to particular applications.

There is a lucid description of the basic principles of light measurement, written in a language understandable to the non-specialist reader. It also grabs the nettle and presents mathematical formulae in such a way that even these should be understandable to the non-mathematician. There is a section which deals with characteristics and limitations of measurement devices: optical radiation detectors, calibration of light sources and detectors, and techniques for spectroradiometry and broad-band radiometry. Although repetitious in parts, there is a lot of useful information, some of it fairly advanced but these chapters comprise essential reading for anyone contemplating carrying out the sort of studies described in the rest of the book. There are then chapters describing action spectroscopy and application of lasers in photobiology and photochemistry. These contain further descriptions of detector types which seem a little out of place and only a little on action spectroscopy *per se*, which is a pity. The discussion of photochemistry using time resolved techniques is fascinating but would certainly require some prior knowledge on the part of the reader. The final chapters outline some very interesting topics: ultraviolet radiation dosimetry with polysulphone film, computer programs for estimating ultraviolet radiation in daylight and optical radiation interactions with living tissue. These are all well written and useful contributions.

The standard of this work is very high. It is not a beginners' guide, but the reader who is prepared to give the extra effort required to understand the material with which he or she is unfamiliar will find this a rewarding experience. This is a book which can be unequivocally recommended to anyone with serious intent on radiation measurement in photobiology.

H.MOSELEY

Dermabrasion and Chemical Peel: A Guide for Facial Plastic Surgeons. E.G.MCCOLLOUGH (1989). New York: P.Royal Langsdon Thieme Medical Publishers, Inc.

This is a remarkable and detailed book on dermabrasion and chemical peel written by a plastic surgeon jointly with a dermatologist, or dermatological surgeon. It certainly would not have wide appeal to many British dermatologists at the present time but it may well be that future development in British dermatology may find such publications of great interest. Some of the before and after photographs, particularly with regard to phenol peels, are most convincing, and as more and more patients demand cosmetic procedures rather than come to us with straightforward dermatological disease, it may well befit us to read such manuals with care. There is, unfortunately, a good deal of use of United States trade names for soaps, proprietary creams etc. and the spelling leaves a good deal to be desired. There is also a very sketchy description of risks to the surgeon and his assistants from viruses during the procedure of dermabrasion which I would have thought merited expansion and considerably more discussion. The authors are firmly in favour of phenol versus trichloracetic acid facial peels, despite the hepatotoxic, nephrotoxic and cardiotoxic effects of phenol absorbed through the skin. They regard it as mandatory that there should be cardiac monitoring and an intravenous line set up for any procedure involving more than a third of the face area. To British minds this will seem like overkill (to coin a phrase), particularly when the authors write that they have not yet seen a case requiring cardiac resuscitation, although some arrhymthias are not uncommon. Many will also question on this side of the Atlantic whether it is justified to perform a cosmetic procedure requiring such stringent precautions. Clearly there is room for a good debate on this and other topics covered in this volume.

J.S.COMAISH

Illustrated Cutaneous Laser Surgery—A Practitioners Guide. J.S.DOVER, K.A.ARNDT, R.G. GERONEMUS, S.M.OLBRICHT, J.M.NOE AND R.S.STERN (1990). East Norwalk, Connecticut: Appleton and Lange. 142 pages, 129 figures and illustrations. ISBN 0-8385-1432-4. Price $98.00.

The purpose of this book is to provide a practical approach to the use of lasers in the treatment of skin disease, the authors are mainly from the Harvard Medical School in Boston and have produced a 'cook book' approach with how to choose a laser and a practical guide to laser surgery. It is intended for dermatologists, plastic surgeons, ENT surgeons, primary care physicians and chiropodists. There are seven chapters and a comprehensive index covering physics of lasers, CO_2 lasers, Argon lasers, Dye lasers, Neodynmium-YAG lasers, photodynamic therapy and choosing a laser to treat vascular abnormalities. Each chapter has a bibliography and appendix with a list of clinical applications together with a patient programme instruction sheet which it is suggested can be photocopied and handed to a patient.

There are many excellent colour and black and white illustrations of before and after therapy, but some are not strictly comparable and the results are not uniformly good.

Lasers in Britain are mainly used in the treatment of port wine stains and angiomas. I recently priced a tunable Dye laser which was estimated at £139,000. It is therefore unlikely that many British dermatologists or plastic surgeons will have the opportunity to use this form of treatment.

The book, however, is extremely well laid out and easy to read and gives a clear and comprehensive guide to their use in the United States of America, where they appear to be indicated in a wide range of dermatoses including pigmented naevi. The book is recommended for the departmental library and is good value for money.

N.MORLEY

Skin Manifestations of AIDS. N.S.PENNEYS (1990). London: Martin Dunitz; ISBN: 1-8531-7013-5. Pp. 210. £39.95.

Dr Penneys' style is trenchant, his references extensive and illustrations excellent. He has therefore achieved the three goals that he set himself in the Preface.

The subject is covered logically but the inclusion of chapters on mucosal lesions and paediatric AIDS means that some ground is covered twice. Several important points are made clearly. Common infections, for example, may be very extensive or appear in uncharacteristic patterns. Rare infections may be manifest and I for one was unaware of alternariosis. Another difficult area is the dual or triple infection: here the clinical appearances are unlike those of the individual diseases. Kaposi's sarcoma has protean manifestations. Fine colour pictures are shown of confusing presentations, e.g. barely visible pink/purple macules, perifollicular papules and an appearance akin to lupus pernio.

There are few errors, however in a series of pictures on page 160 a lesion is shown to progress through three stages but on the third shot the limb has also changed! Three similar publications have appeared in the last year and each is considerably cheaper than this volume; however, it is an excellent book.

G.B.COLVER

Nickel and the Skin: Immunology and Toxicology. Edited by H.I.MAIBACH AND T.MENNÉ (1989). Boca Raton: CRC Press, Inc. Pp. 223. ISBN 0-84993-6976-2. Price £79.50.

Nickel is a most important metal in medicine and in surgery. For years nickel has been the commonest sensitizer in women and in some clinics the incidence in men is increasing. Nickel is a significant allergen in orthopaedic surgery, it is an industrial sensitizer and an industrial carcinogen. Its role in the chronicity of hand eczema in sensitized women remains controversial.

This book covers every aspect of medical interest in nickel. It is divided into 19 chapters each written by an author with a particular interest in the subject. Each contribution is short and informative. The initial chapters deal with chemistry, exposure, carcinogenicity and alloys and coatings. These are followed by immunology, testing, genetics and epidemiology. The last eight chapters discuss occupational dermatitis, hand eczema, nickel in food, drug interaction, orthopaedic surgery and dentistry. A final chapter discusses the influence on sensitization of culture, education and economic factors. Finally the editors emphasize that nickel dermatitis is a preventable disease.

This book is well designed, the information is well presented and easily accessible. It will be of value and I recommend it to dermatologists, occupational physicians, immunologists and toxicologists.

E.CRONIN

Book Received

The Biological Basis of Disease. Selected papers by P.R.J.Burch. Edited by M.S.Chesters and J.E.Burch. Published by Leeds University Press 1989. ISBN 0 85316 147 X.

British Journal of Dermatology (1990) **122,** 850.

News and Notices

New Concepts in Cutaneous Laser Surgery
Boston U.S.A., 27–28 October 1990

The Department of Dermatology of the Beth Israel Hospital and Harvard Medical School, Boston, Massachusetts, are jointly sponsoring the course 'New Concepts in Cutaneous Laser Surgery' on 27 and 28 October 1990, at the Hyatt Regency Hotel in Cambridge, Massachusetts. The first day of the course will address the theoretical and practical aspects of cutaneous laser surgery. During the second day, all participants will be offered hands-on experience with various types of instruments, including argon, continuous wave and pulsed dye, carbon dioxide, neodymium: YAG, and copper vapour lasers. The course will include formal lectures, panel discussions, and videos of demonstrations of laser techniques and laser safety. For further information contact: Department of Continuing Medical Education, Harvard Medical School, 25 Shattuck Street, Boston, MA 02115, U.S.A. (617) 732-1525.

Fifth Pan African Congress of Dermatology
6–9 February 1991, Nairobi, Kenya

The fifth Pan African Congress of Dermatology will be held in Nairobi from 6–9 February 1991. The main topics to be covered are on AIDS and other sexually transmitted disorders, leprosy, tropical dermatology and allergic dermatoses.

For further information contact: Dr Melanie Miyanji, Organizing Secretary, PO Box 20714, Nairobi, Kenya.

Training Course in Contact Dermatitis
Birmingham, 1–5 October 1990

A 5-day residential course to cover all aspects of Contact Dermatitis is to be held at the Institute of Occupational Health, University of Birmingham and at The Skin Hospital, Birmingham from Monday 1 October to Friday 5 October 1990.

Further details may be obtained from Mrs Joyce Blake, Course Co-ordinator, Institute of Occupational Health, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

British Inflammation Research Association

The British Inflammation Research Association (BIRAs) is a new scientific society which fosters links between research workers in industry and academic groups working on all aspects of inflammatory disorders. One of the main activities of the Association is holding 1-day scientific meetings on different aspects of research on inflammation. Those who are interested should write to the Treasurer of BIRAs, Dr F.B.de Brito, Rhône-Poulenc Ltd, Biological Research, Rainham Road South, Dagenham, Essex RM10 7XS, U.K.

University of Liverpool

Dr P.S.Friedmann, Senior Lecturer in the Dermatology Department of the University of Newcastle upon Tyne, has been appointed to the newly created Chair of Dermatology in the University of Liverpool.

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Foreword

The Hotel La Bobadilla, located in a remote place in the south of Spain, was the venue for a meeting from the 26–28 February, 1990, of 30 dermatologists, nephrologists, renal physiologists and pathologists from all over the world. The subject of discussion was whether or not cyclosporin A (Sandimmun®) can be recommended for the treatment of patients with psoriasis. Three days of lively sessions, lasting from early morning until late at night, resulted in a consensus on who, who not, and how psoriatic patients should be treated with cyclosporin A.

On behalf of all the participants of this important event, we would like to thank Sandoz Pharma Ltd in Basle, Switzerland for their generous support which made this workshop possible. We hope that the readers of this supplement will also share our appreciation.

M.J.MIHATSCH
K.WOLFF
Hotel La Bobadilla
Loja, Granada, 1990

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A consensus report: cyclosporin A therapy for psoriasis

Human studies, using oral cyclosporin A (CyA; Sandimmun®) therapy for psoriasis, have been in progress for nearly 7 years. Cumulative data on the clinical effects of this therapy in 600 patients have been collected and are presented in this supplement. These data are derived from multiple open and controlled studies. The efficacy of this treatment in psoriasis is well recognized, and the safety of short-term treatment, up to 1 year, is also established, provided that guidelines for therapy are observed. However, there is concern regarding the long-term safety of CyA treatment of psoriasis as experience is still limited. Careful monitoring of all CyA-treated patients is therefore *mandatory* and CyA should only be used by dermatologists who have access to expertise in the use of CyA and have the appropriate facilities for monitoring the long-term risks.

Who should be treated?

Patients considered for this form of treatment should have psoriasis of such severity that the risks inherent in this treatment, as outlined in this supplement, are acceptable in that patient.

Who should not be treated?

Psoriatic patients with other systemic disorders and who are taking additional medications should be carefully reviewed before prescribing CyA because of possible drug interactions and other adverse effects. In particular, this applies to concomitant therapy with potentially nephrotoxic and cytotoxic agents. Patients receiving concomitant immunosuppressive or radiation therapy (including PUVA and UVB) should not receive CyA because of the possibility of overimmunosuppression and the risks of infection and malignancy. Patients should also be warned of the risks of overexposure to solar radiation. Drugs which interfere with the bioavailability of CyA should be avoided because of the danger of unpredictable CyA blood levels. Also, agents known to be nephrotoxic, such as aminoglycoside antibiotics and non-steroidal anti-inflammatory drugs, should be avoided.

Patients who have the following conditions should, in general, not be considered: previous or concomitant malignancies; infections of any type; primary or secondary immunodeficiency; severe chronic organ dysfunction; or drug or alcohol abuse. Patients with abnormal renal function or uncontrolled hypertension as well as those who are uncooperative should not be accepted for CyA therapy.

How should the treatment be performed?

The patient should be carefully instructed concerning the nature of the treatment. The starting dose should range from 3 to 4 mg/kg body weight. The drug can be taken as a single daily dose or divided into two daily doses. If improvement does not occur, the CyA dose can be increased, but should not exceed 5 mg/kg body weight/day orally. If there is considerable improvement of skin lesions, the dose should be reduced stepwise to ascertain the individual's minimum maintenance dose requirements at which serum creatinine remains within the accepted range (see below). Dose adjustments should always be made in steps of 0·5–1 mg/kg body weight.

If adverse effects arise and depending on their severity, the daily dose should either be reduced in steps or, if necessary, treatment should be discontinued. Special attention should be paid to serum creatinine increases of more than 30% over the *patient's own baseline values* as confirmed on two or more occasions within 2 weeks. In the absence of relevant data, hypertension (diastolic blood pressure over 105 mm Hg or a sustained diastolic blood pressure over 95 mm Hg) should be treated according to accepted standards of care and, in this setting, diuretics and beta-blockers should not be used.

Cyclosporin A treatment should be discontinued in cases of insufficient response of psoriatic lesions with CyA 5 mg/kg body weight within 6 weeks, or where the effective dose is not compatible with the safety guidelines (see above).

Present experience indicates that severe psoriasis recurs when CyA treatment is stopped. The goal of maintenance therapy is to keep the patient as lesion-free as possible with the least amount of CyA delivered, and total clearing of the skin should not be the final goal. After reaching a relatively disease-free state, the patient should be given the minimum effective maintenance dose (see above). If patients have been successfully treated for 1 year, any decision to extend therapy should be taken in the context of information available, as the data regarding long-term use of CyA in psoriasis is still being accumulated.

If a patient experiences a serious flare during the maintenance treatment, therapy can be changed to a dose that is sufficient to control psoriasis while remaining compatible with the safety guidelines. Every attempt should then be made to reduce the dose to the lowest effective level.

How should the patient be monitored during therapy?

Psoriasis is not a life-threatening disease, and the use of a potentially harmful drug such as CyA must be carefully monitored and controlled. Thus, careful dermatological and physical examinations, including blood pressure measurements (on at least two occasions before initiating therapy) should be performed. As CyA is immunosuppressive, a search for all forms of tumours, including those of the skin and cervix, should be carried out. In order to limit nephrotoxicity, control must be directed towards (a) exclusion of patients with potential risk factors; (b) limiting the dose to a maximum of 5 mg/kg body weight and attempting to adhere to the lowest possible dose; (c) careful monitoring of renal function.

Prior to therapy, a fasting serum creatinine should be obtained on at least two separate occasions to ensure accurate baseline values. There is controversy as to the ability to identify early, reversible CyA nephrotoxicity by measuring serum creatinine only. Renal function assessment [for example, glomerular filtration rate (GFR), using inulin, isotope or iothalamate clearance or renal blood flow] should, if possible, be made at baseline. It is *mandatory* that the serum creatinine be measured frequently and carefully monitored for any deterioration. Ideally, the GFR (or equivalent) should be repeated within 2 months of initiation of therapy and correlated with changes in serum creatinine. If the two measurements are discrepant, more frequent monitoring of the GFR is indicated. Cyclosporin A dosage should be adjusted or discontinued to prevent the serum creatinine from rising to more than 30% above the *patient's own baseline values*. Initial and periodic investigations of bilirubin, liver enzymes, serum potassium, magnesium, uric acid and urinary protein are required.

Regular clinical and laboratory examinations are mandatory. Follow-up assessment, especially of blood pressure and serum creatinine, should be performed every 2 weeks during the initial 3 months, and then monthly if the patient is stable, unless dosage adjustments are made. Adverse effects, such as tremor, hypertrichosis, gingival hyperplasia and nausea, should be noted. Because of its poor predictive value in psoriatic patients, routine measurement of drug blood levels is not essential, but may be of value in cases of possible drug interactions. A renal biopsy is not necessary in uncomplicated cases.

SAFETY GUIDELINES FOR CYCLOSPORIN A THERAPY IN PSORIASIS

- The minimum effective dose should be used and this should not exceed 5 mg/kg body weight/day.
- Serum creatinine and blood pressure should be monitored regularly.
- If the creatinine increases by more than 30% over the *patient's own baseline values*, the dose should be decreased by 0.5–1 mg/kg body weight and, if there is no significant improvement, CyA therapy should be stopped.
- Sustained hypertension should be treated or CyA therapy be reduced or stopped.
- Co-medication with drugs known to interfere with the bioavailability or action of CyA should be avoided.

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Mechanism of action and rationale for cyclosporin A in psoriasis

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SUMMARY

Cyclosporin A (CyA) is the forerunner of a new generation of immunosuppressive drugs. It is highly effective in both antibody- and cell-mediated immunity and in inhibiting chronic inflammatory reactions. Cyclosporin A is, therefore, widely used to prevent rejection in clinical organ transplantation and appears to be very promising in the treatment of several autoimmune diseases, in particular psoriasis. Cyclosporin A is not only effective when given at the time of sensitization (prevention of rejection), but also when administered therapeutically during an immune response, such as during the active course of an autoimmune disease. The most important aspect of its immunosuppressive mechanism is the inhibition of lymphokine production secreted by activated T cells. The pharmacological effects of CyA are rapid in onset, dose-dependent and often quickly reversible when treatment is stopped.

Cyclosporin A (CyA) is a cyclic undecapeptide isolated from *Tolypocladium inflatum Gams.* The compound has several N-methylated amino acids and one novel C_9-amino acid. The peptide is neutral, rich in hydrophobic amino acids, and insoluble in water, but soluble in lipids and in many other organic solvents. Studies revealed that it was effective in inhibiting both humoral and cell-mediated immune responses. Its effect was reversible and specific for T lymphocytes. Haemopoietic tissues were not affected in immunosuppressed animals.

ANTIBODY-MEDIATED IMMUNITY

The potent blocking effect of CyA on primary antibody production against sheep erythrocytes has been well documented in several species. Although the optimal doses for suppression of antibody synthesis are approximately twice those for inhibiting cell-mediated immunity, the therapeutic index of CyA is significantly superior to that of comparable immunosuppressants such as azathioprine, cyclophosphamide and methotrexate. In addition, its inhibitory action is not primarily due to cellular depletion as observed with these cytostatic drugs. Both IgM and IgG isotypes are suppressed in the primary response. Time course studies show that the drug

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acts on the early events that precede mitosis in the immune response. Especially noteworthy is the rapid and complete reversibility of its suppressive effect, which is independent of the duration of treatment.

When comparing the effects of CyA against various antigens, it is evident that primary antibody formation against thymus-dependent (TD) and thymus-independent (TI-2) antigens, but not TI-1 antigens, is easily suppressed. Whether CyA affects B cells directly or indirectly through suppressing T helper (T_H)-cell function has been investigated using various experimental protocols. The emerging consensus indicates that primary TD and TI-1 B cells, and primed T_H cells are CyA-resistant whereas primary TI-2 B cells and non-primed T_H cells are CyA-sensitive. Furthermore, it has been demonstrated, using viral antigens, that the TD switch from IgM to IgG isotope is suppressed by the drug.

Although CyA has been shown to be effective in inhibiting an ongoing humoral response in models using repeated blood transfusions to elicit alloantibodies and in several autoimmune models with overt autoantibody production, such a claim must be limited, as in some other models the drug has demonstrated no effect on continuous autoantibody synthesis. Some experiments clearly suggest that CyA inhibits priming *in vivo* while others do not. By assessing rats presensitized with allogeneic spleen cells but without receiving CyA, and which were subsequently challenged with donor-specific blood transfusions, treatment with the drug was found to prevent the anamnestic alloantibody response. In experimental myasthenia gravis, the inhibition by CyA of a secondary autoantibody response was dependent on the challenging antigen dose.

There is no doubt that the secondary humoral response remains somewhat refractory to the immunosuppressive action of CyA. However, there is also evidence that significant reduction in secondary antibody titres may be obtained under certain experimental conditions, provided that high doses of the drug and relatively low antigen amounts for the challenge are used.

CELL-MEDIATED IMMUNITY

Transplantation of allogeneic organs

A wide range of different organs and tissues have been transplanted between various species, and their survival, except for xenografts, has been successfully prolonged with the use of CyA. These experimental grafts can be divided into five categories: classical allografts; new allografts which have seldom or never been attempted before the availability of CyA; composite grafts comprising more than a single organ; xenografts in which the rejection process may qualitatively differ from that involved in an allograft rejection; and bone marrow grafts whereby the entire immune system is replaced, thus causing a graft-versus-host (GvH) reaction.

Attempts to achieve synergism in variously experimentally combined models and the respective effects of each suppressive component on the mechanism promoting unresponsiveness have been analyzed. Obviously, the most promising protocols are those in which each suppressive component is complementary to another – with each acting on a different step in the activation cascade.

Several factors which eventually reduce immunogenicity may contribute to the relative adaptation of a graft, including replacement of the endothelium of the graft, which is a potential source of antigen-presenting cells (APC), by the host's own cells, and suppression of lymphokine production which, in turn, inhibits activation of effector cells by preventing expression of interleukin-2 receptors (IL-2R) induced by IL-2. Inhibition of MHC (major histocompatibility complex) Class I and Class II products stimulated by gamma interferon (γ-IFN), leaves the specific suppressor mechanisms intact and thereby reduces the function of APCs to below the critical level. Cyclosporin A appears to contribute directly or indirectly to these factors and monotherapy with this drug at remarkably low doses is effective in maintaining long-term allograft acceptance.

Delayed-type hypersensitivity (DTH)

Cyclosporin A inhibits both the induction and effector phases of the DTH response in several species, including man. This suppressive effect is apparently independent of the type of antigens used (chemicals, proteins, virus or spleen cells).

Both the primary and secondary DTH reactions were eliminated with appropriate treatment with CyA; suppression of the secondary response was independent of modulation of the primary response. If the DTH reaction was elicited by injections of activated, antigen-specific, cloned T_H cells, CyA given at up to 10, but not more than 15, hours later was efficacious in preventing the reaction. In contrast, when the challenge was induced with antigen, the effector phase was inhibited only if the drug was given before or at the same time as the antigen. Topical CyA application at the site of antigen administration also prevented the effector phase of the DTH reaction. Suppression by the drug is reversible and of short duration, as indicated when a secondary challenge 96 hours after the primary response elicited a positive DTH response.

The target cells for the action of CyA in the DTH response are those mediating the specific or immunological component of the reaction as the agent has no direct effects on the non-specific, acute inflammatory component. Various studies have demonstrated that the DTH reaction could be adoptively transferred by CD5 non-adherent T lymphocytes, the cells affected by CyA. These cells also release lymphokines *in vitro* when cultured with the relevant antigen. Cyclosporin A interferes with the DTH reaction by presumably suppressing the release of lymphokines and other mediators of inflammation. The addition of rIL-2, but not of γ-IFN, reverses the inhibitory action of the drug.

The finding that CyA does not interfere with the clonal expansion of antigen-specific DTH-mediating cells implies that DTH precursors are not sensitive to CyA inhibition. It has been suggested that the effector functions of the T_H and T_{DTH} lymphocytes are suppressed by the drug while their proliferative capacity is resistant. This has been confirmed in other transfer experiments which showed induction of T_{DTH} effector cells to be insensitive to CyA while the effector function of this T_{DTH} subpopulation remained CyA-sensitive.

AUTOIMMUNITY

The capacity of a drug to modify the course of an autoimmune disease can be assessed either preventively—its ability to inhibit the primary event of sensitization—or therapeutically—its ability to alleviate the symptoms of the ongoing disease after sensitization.

Cyclosporin A has been studied in many experimental autoimmune models. Although animal models can provide some understanding of autoimmunity, extrapolation to man must be with caution. Experimental models of autoimmune diseases have the disadvantage of often being artificially induced and correlation with the human counterpart, even of the genetic models, is disputed. Thus, while both the induced and spontaneous models of uveitis and nephritis are susceptible to CyA treatment, only the induced, and not the spontaneous, models of thyroiditis respond to therapy. Similarly, streptozocin- and virus-induced diabetes are refractory to the drug whereas spontaneously developing diabetes both in bio-breeding (BB) rats and non-obese diabetic (NOD) mice reacts promptly to prophylactic treatment with CyA.

Experimental models of arthritis

Freund's adjuvant arthritis (FAA) in rats is an experimentally induced condition with T cell-dependent autoimmunity which bears a close resemblance to several forms of human arthritis. Injection of mycobacterial adjuvant leads to T-cell activation and proliferation and, subsequently, to massive mononuclear cell infiltration of the joints which ultimately results in complete joint destruction. Cyclosporin A strongly reduced the chronic inflammatory symptoms when given either preventively or therapeutically. Unlike most anti-inflammatory drugs, CyA does not induce stomach ulcers in the rat.

Because of evidence suggesting inflammation-mediated osteopenia in the rat, the therapeutic effect of CyA arthritis-related bone resorption in rats with FAA was investigated. Treatment with the agent resulted in a significant dose-dependent regression of articular swelling and cartilage degradation concomitant with improvement in bone density; the protective effect of the drug against articular damage was supported by a progressive improvement in total epiphyseal glycosaminoglycan content. Cyclosporin A-induced blockade of T-cell activation via inhibition of lymphokine production interrupts the immune-mediated inflammatory process and, in turn, leads to a reduction of phlogogenic cytokine release of IL-1, for example, which is likely to be a mediator of bone and cartilage breakdown in arthritic disease. Cyclosporin A has also been used in the naturally occurring retrovirus-induced caprine arthritis model in which advanced symptoms, especially severe inflammation-induced damage to the knee joints, were reversed, as demonstrated by radiography, with continuous drug treatment.

Collagen arthritis can be readily induced in many strains of rats by immunization with heterologous or homologous native type II collagen emulsified in incomplete Freund's adjuvant. This disease, characterized by the development of both cellular and humoral immune responses to type II collagen, can be transferred by sensitized spleen and lymph node cells as well as by IgG antibodies to type II collagen.

These findings are consistent with the proposal that collagen arthritis is the result of immunological hypersensitivity to type II collagen. The pathogenic difference between collagen arthritis and FAA with the use of CyA was assessed in a study involving daily treatment with the drug for 14 days. This regimen completely suppressed the development of collagen arthritis and FAA in rats during the observation period. To determine whether the unresponsiveness produced by the drug was antigen-specific, the CyA-protected rats were rechallenged with either type II collagen or complete Freund's adjuvant (CFA) after discontinuation of the drug. Type II collagen-immunized CyA-protected rats did not develop arthritis in response to re-immunization with type II collagen, although FAA developed in response to a subsequent injection of CFA, and CFA developed in response to a subsequent injection of FAA.

These results indicate that specific immunological unresponsiveness can be induced by CyA in these two experimental models of polyarthritis, and that there is no cross-reactivity between type II collagen and the mycobacterial cell wall components. The anti-arthritic effect of CyA is mediated by systemic and local effects on the immune system as reduction of the disease is associated with a significant decrease in serum antibody against type II collagen and a decrease in lymphocyte accumulation in arthritic joints.

The effects of CyA on collagen-induced arthritis have also been studied in mice. Prophylactic treatment suppressed the development of arthritis and the immunological response to native type II collagen. Treatment with CyA (21–34 days), started on the same day as the booster injection with type II collagen (re-inoculation is required to maintain a high incidence of arthritis in mice), also inhibited the development of arthritis and immunity to collagen.

Injection of an aqueous suspension of cell wall fragments from group A streptococci and other bacteria into susceptible rats induced an acute, self-limiting polyarthritis followed by a chronic proliferative, erosive polyarthritis and hepatic granulomas. The effects of CyA were evaluated despite the controversy regarding the role of T lymphocytes in this model. Continuous daily treatment with CyA for 6 weeks, started either 1 day before or 7–12 days after streptococcal injection, resulted in a significant inhibition of the chronic proliferative erosive synovitis (but not of the acute arthritis) and total inhibition of the hepatic granulomas. On stopping the drug at 6 weeks, its effect continued for at least 5 more weeks, demonstrating long-term benefit without continuous administration of the drug. In contrast, CyA given only during the acute phase of the experimental disease (1–12 days) did not inhibit the development of the chronic disease. These data are evidence of an important role for the T_H cells in this model.

Experimental models of nephritis

Immunologically induced experimental models of nephritis to assess the effect of CyA included: acute serum-sickness nephritis in rabbits; chronic serum sickness in rats; anti-glomerular basement membrane nephritis in mice; *in-situ* immune-complex glomerulonephritis in rats; chronic glomerulo- or Heymann's nephritis in rats; tubulointerstitial nephritis in rats; and mercuric chloride-induced autoimmune glomerulonephritis in rats. In addition, the effect of the drug in the spontaneously developing autoimmune lupus model in the New Zealand black-and-white (NZB/W) hybrid mouse has also been evaluated. Cyclosporin A treatment was effective in both preventing the development of nephritis in all of the models tested and in improving the symptoms of established nephritis in most models, except for Heymann's nephritis and the antibody-induced glomerulonephritis. It appears that the drug interfered mainly with cellular–effector mechanisms involved with the proteinuria, as a rapid reversal of proteinuria was obtainable in several instances despite the persistence of autoantibodies and circulating immune complexes. However, preventive CyA treatment was also effective in suppressing autoantibody formation. Treatment with the drug induced a state of unresponsiveness in Heymann's nephritis and in mercuric chloride-induced glomerulonephritis.

Psoriasis: a hypothesis

Psoriasis is a relatively common chronic disease of unknown aetiology, although there is increasing evidence that it is perpetuated through an immunological mechanism. Studies directed at the detection of a biochemical defect in psoriatic skin have revealed a number of abnormalities related to transmembranous signal-transducing systems. The abnormalities of the histogenetically different cell populations present in psoriatic skin lesions include an increase in and activation of many cell types, especially immunocytes. The immune system of the skin shows an increase in subepidermal dendritic cells and epidermal infiltration by a large number of T cells; a wide variety of mediators (interleukins and interferons) may also play a role in keratinocyte hyperproliferation and inflammation. The immunogens responsible for these events, however, have not been identified.

There is no experimental animal model of psoriasis. Although phorbol ester-treated mouse skin is inadequate, the epidermal hyperplasia, vascular alterations and neutrophil accumulation as seen in psoriasis occur. Drugs with anti-psoriatic action, such as corticosteroids, retinoids and CyA, are effective in both. Protein kinase C activation occurs for reasons not yet known both in psoriasis and in the mouse model. However, the latter is an acute inflammatory reaction which does not involve the immune system. Nevertheless, topically applied CyA strongly inhibits the phorbol ester-induced ornithine decarboxylase activity by blocking its mRNA accumulation. Cyclosporin A also inhibited approximately 50% of the stimulation of IL-1_α mRNA accumulation by phorbol ester. Histology of the treated mouse skin 5 hours after phorbol ester and CyA application revealed that the drug had prevented neutrophil infiltration of the epidermis, suggesting that no potent chemotactic stimulus within the epidermis had occurred.

MECHANISM OF ACTION OF CYCLOSPORIN A IN AUTOIMMUNE DISEASES

Effect at the cellular level

Most, if not all, of the immunosuppressive effects of CyA are the consequence of the inhibition of IL-2 and other lymphokine secretion by activated T cells. At a cellular level, this implies that:

1. Cytotoxic and other effector T cells that destroy the target cells by interaction with the antigen are inhibited. In autoimmune diseases, this prevents tissue destruction.
2. The lymphocytic subpopulation of T-helper cells no longer produces and secretes IL-2 nor the lymphokines necessary to activate B cells and their maturation into antibody-secreting (therefore autoantibody-producing) plasma cells.
3. Inflammatory effector mechanisms cease to function in the absence of other T_{DTH}-originated lymphokines, such as macrophage-activating factor, interferons and colony-stimulating factors. In autoimmune diseases, this inhibits the inflammatory process.

These effects are all rapid in onset, dose-dependent and often quickly reversible on stopping treatment. Other CyA effects on the immune system are less well-defined and controversial; these include: inhibition of IL-1 production; possible inhibition of IL-2R expression on membranes; failure to affect T-suppressor cell function; and direct inhibition of activated cytotoxic T cells. It is important that CyA does not affect the main macrophage and granulocyte functions, such as phagocytosis, chemotaxis and the release of most monokines, as this suggests that the drug is more selective than other immunosuppressants currently in use.

Effect at the subcellular level

Certain clinical benefits with CyA can only be explained by assuming an effect of the drug at a specific site on the chromosomes associated with lymphokine production. Cyclosporin A most probably manifests its immunosuppressive effects by inhibiting the synthesis of IL-2 and other lymphokine mRNAs during the process of T-cell activation. Effects on the other events of the activation process, for example IL-2R expression, are not clear. The detailed molecular events involved in CyA inhibition of mRNA synthesis are not known, but it is likely that the drug prevents transduction of the mitogenic signal from the cell surface to the nucleus at a time subsequent to the rise in intracellular calcium generated by the mitogenic signal. Its immunosuppressive action also appears to be related to the binding of an intracellular receptor protein, cyclophilin, a major cytosolic constituent of all cells. The normal physiological function of this protein and its involvement in T-cell activation, if any, are not yet known. The hypothetical mechanism of CyA action at the subcellular level (Table I) is illustrated in Figure I.

TABLE 1. Hypothetical mechanism of action of CyA

Cyclosporin A does not inhibit recognition of antigen by the T-cell receptor.

Cyclosporin A has no effect on transmembrane signalling. Mitogen- or antigen-induced activation of protein kinase C and phosphoinositol turnover with the subsequent rise in intracellular Ca^{2+} levels are not inhibited.

Cyclosporin A most likely diffuses passively through the cell membrane and is concentrated intracellularly where it is bound to cyclophilin, a novel, ubiquitous, 18 kD CyA-receptor protein which exerts prolylpeptidyl-*cis*-trans-isomerase activity and is CyA-sensitive. The substrate for this enzyme is unknown, but it is most likely involved in the activation process eventually by interfering with nuclear factor-binding at regulatory sites of specific genes.

Cyclosporin A has been shown in murine and human cell lines and in normal lymphocytes to inhibit transcription of mRNA for a variety of lymphokines. However, it does not inhibit the greater part of mitogen-induced protein synthesis.

Cyclosporin A inhibits the synthesis of IL-2R only at high concentrations.

Synthesis of other activation antigens and expression of MHC gene products are significantly reduced by CyA.

The effect of high concentrations of CyA on the function of APCs, such as the processing and presentation of antigen to T cells, and IL-1 production, is still controversial.

CONCLUSIONS

Cyclosporin A is a potent immunosuppressive compound that is effective in prolonging allograft survival and in the treatment of many autoimmune syndromes. Its pharmacological effects on both antibody- and cell-mediated immunity are rapid in onset, dose-dependent and

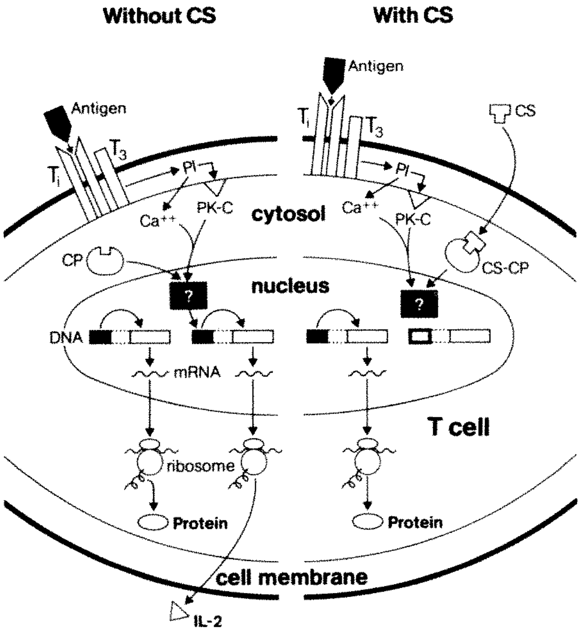

FIGURE I. Hypothetical mechanism of action of CyA and its effects on T-cell activation. CS, CyA; CP, Cyclophilin; PI, Phosphoinositol turnover; PK-C, Protein kinase C; IL-2, Interleukin 2; mRNA, messenger RNA.

quickly reversible. Its suppressive action is primarily directed towards activated T-cell functions, especially secretion of lymphokines. Large series of experimental studies have revealed that its mechanism of action *in vivo* may differ from and is more complex than that observed under straightforward *in vitro* conditions.

Cyclosporin A has shown remarkable efficacy in numerous animal models of autoimmunity when given both prophylactically and therapeutically. On the basis of the vast experience gained with transplant patients, many clinical studies of autoimmune diseases have been performed or are still ongoing. Pilot studies, often conducted in therapy-resistant patients, have been followed-up by randomized controlled trials with a shift towards patients with less advanced disease and frequently involving multiple centres for adequate statistical power.

As CyA is known for its selective inhibition of T-lymphocyte functions and as there is increasing evidence that psoriasis is a T cell-mediated disease, there is a sound basis for use of the drug in psoriasis. Oral as well as intralesional treatment with CyA has been shown to be highly effective in psoriasis, although the precise mode of action of the drug in this indication is still unknown. Low doses of CyA (3–5 mg/kg/day) are effective in clearing and maintaining improvement over a long period of time. When the drug is stopped, gradual relapse occurs in all patients, but there is no rebound. However, topical CyA, which would greatly diminish its side-effects, has not yet been shown to be effective.

REFERENCE

All of the studies and conclusions referred to in this paper can be found in the following reference.

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Four years of experience with cyclosporin A for psoriasis

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SUMMARY

Forty-four patients with severe psoriasis have been treated with cyclosporin A (CyA) for 2–50 months (mean 17 months). During the study, 31 (70%) of these patients achieved a >70% reduction in PASI score, 39 (88%) achieved a >60% reduction and 42 (95%) a >50% reduction. The mean initial dose of CyA was 3 mg/kg/day and the mean dose was 3·3 mg/kg/day throughout the study. Twenty-five (57%) patients were maintained on ≤3 mg and six (14%) required >5 mg/kg/day for limited periods to obtain significant improvement. In three of these patients, this was achieved with 6 mg/kg/day but, of the remainder, one required 7 mg and two required 10 mg/kg/day. Of the 44 patients, 32 (73%) are still taking CyA. Patients were discontinued because of: side-effects directly attributable to treatment ($n = 4$); remission of psoriasis ($n = 4$); death ($n = 1$); defaulting ($n = 1$); infrequent attendance ($n = 1$); high doses of NSAID were necessary for arthritis ($n = 1$). Before starting CyA, 39 patients were normotensive; 21 (54%) developed mild hypertension. In 28 patients where the GFRs were estimated before and during treatment, there was a 16% reduction ($P < 0{\cdot}0001$) during a mean period of 8 months. Two patients developed malignancies. The incidence of hypertension and percentage decrease in GFR were strongly correlated with the dose required to control the psoriasis.

Studies during the early part of the 1980s at St Mary's Hospital implicated activated CD4 cells in the pathogenetic mechanism of psoriasis and resulted in the hypothesis that psoriasis is a disorder of abnormal keratinocyte proliferation mediated by T cells.[1] Cyclosporin A was also predicted to be an effective drug in the treatment of psoriasis, and the results of early trials have supported this expectation.[2–4] The main action of CyA is the inhibition of cytokine production in the activated CD4 cell, particularly of IL-2, a T-cell growth factor. After more than 4 years of using CyA in the treatment of psoriasis, this is a report of the experience with this therapy.

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METHODS

Patients

A total of 44 patients (24 males and 20 females) aged 26–76 years (mean 48 years) has been treated with CyA. Patients selected were those who failed to show adequate improvement with topical therapy and/or psoralen and long-wave ultraviolet light (PUVA), and oral drugs including methotrexate, etretinate and hydroxyurea. The duration of treatment has ranged from 2–50 months (mean 17 months). Three patients have been treated for over 4 years, 11 for over 3 years, 13 for over 2 years, and 21 for more than 1 year.

Dose

Cyclosporin A was taken once daily at night. The average initial dose was 3 mg/kg/day. Patients were seen once a week for the first month and then every 2–6 weeks, depending on the control of their psoriasis, and the incidence and severity of side-effects. The dose was altered by 0.5–1.0 mg/kg/day, depending on the clinical response and/or side-effects.

Clinical assessment

The efficacy of CyA was assessed by measuring the percentage reduction in the Psoriasis Area and Severity Index (PASI) score.[5] Blood pressure was recorded at each visit to the clinic. The upper limit of normal blood pressure was considered to be 160/95 mm Hg.

Liver function tests (serum bilirubin, alkaline phosphatase and aspartate transaminase) and serum creatinine were measured, and a full blood count taken at each visit. The glomerular filtration rate (GFR) was determined by a single injection of ^{51}Cr-EDTA with an SEM of ± 6 ml/min.[6] The normal GFR value was defined as 105 ± 26 ml/min at age 30 years, 98 ± 23 ml/min at age 50 years, and 78 ± 24 ml/min at age 70 years.[7] The GFR was first measured in 13 of the 44 patients after an average treatment duration of 2·5 years (Group I). It was considered to be low in six of these patients, and their CyA treatment was stopped for periods averaging 3–17 weeks. The GFR was then determined before and after starting treatment for periods ranging from 7–26 weeks (mean 15 weeks). In the remaining 31 patients, GFR was determined before beginning CyA treatment (Group 2) and then at 3, 6, 12 and 18 months during treatment. However, if the GFR value was low, the test was repeated earlier than scheduled and after either stopping the drug or reducing the dose.

Cyclosporin A blood levels were measured as whole blood trough levels using radioimmunoassay. During the first 2 years, a polyclonal antibody was used to measure the amount of parent drug and metabolites. The therapeutic range for transplantation was 400–800 ng/ml, above which toxicity was to be expected. During the latter 2 years, a monoclonal antibody that measures only the parent drug has been used. During this period, the therapeutic range for transplantation has been 200–400 ng/ml and levels above this are considered toxic.

The only adjuvant therapy allowed is with topical steroids. Except for three patients who used them on a regular basis, these have been used only occasionally.

RESULTS

All patients considered CyA the best treatment they had received so far on the basis of clinical efficacy and lack of subjective side-effects.

Dosage

The mean average dose for the 44 patients throughout the study was 3·3 mg/kg/day of CyA. Significant improvement was achieved with a mean dose of 3 mg or less in 25 (57%) patients. However, six (14%) patients required more than 5 mg/kg/day in order to control their psoriasis at some time during the study. In three of these six patients, 6 mg/kg/day was sufficient for a clinical response but, in the remaining three, satisfactory improvement was only obtained with 7 mg/kg/day in one and 10 mg/kg/day in the other two.

In these latter three patients, blood pressure rose to hypertensive levels and the GFR decreased by 44%, 45% and 54% in each patient, respectively. These side-effects were considered unacceptable and all three were classified as 'failures'. Their treatment was discontinued despite the fact that, with these relatively high doses, their psoriasis was virtually cleared.

In general, there was considerable variation among patients in the dosage required to achieve a similar clinical response but, once established, this dose and that needed to maintain the improvement tended to be constant in a given patient.

Clinical improvement

The long-term clinical improvement, assessed by the percentage reduction of the PASI score, varied only slightly for individual patients throughout the study, depending on dose and/or activity of the disease. Of the 44 patients, 31 (70%) achieved a greater than 70% reduction in PASI score at some time during the study, 39 (88%) achieved a greater than 60% improvement and 42 (95%) showed a greater than 50% reduction in PASI score. Only one patient was totally cleared of psoriasis; the remainder all had some psoriasis which, for the majority, was considered acceptable.

Current state of patients

Of the 44 patients in this study, 32 (73%) are still taking CyA. The reasons for discontinuing treatment in the other 12 patients were:

1. Four patients cleared sufficiently and did not require further treatment. Although their psoriasis is recurring, it has not yet deteriorated to the extent that treatment needs to be recommenced. The follow-up period is only 3 months.
2. In three patients, the control of the disease could not be achieved by doses below 7 mg/kg/day and, because of side-effects, therapy was discontinued.
3. One patient developed genital warts extending to the groins after 3 months of treatment which was then discontinued.
4. One patient has defaulted.
5. One patient attended the clinic infrequently and did not take the drug as prescribed, and treatment was discontinued.
6. One patient (at age 79 years, the oldest) died of a cerebral haemorrhage.
7. One patient developed severe arthropathy not controlled by CyA at 3 mg/kg/day. Treatment with NSAIDs was instituted by a rheumatologist and CyA therapy was discontinued.

Side-effects

The main side-effects were hypertension and decreased renal function. There were transient, mild and subjective, and did not warrant discontinuing therapy.

Three patients developed malignancies.

Hypertension. Five patients were hypertensive prior to initiating CyA therapy. However, as the hypertension was mild, it was not considered a contraindication as other therapies for their psoriasis had not been effective and/or well tolerated. Of the remaining 39 patients, 21 (54%) developed hypertension during the study treatment while, in nine (23%), their blood pressure was the same or lower at the final assessment.

Overall, for the entire group of 44 patients, the mean blood pressure was 134/86 mm Hg before CyA treatment, and 143/95 mm Hg at the last assessment after treatment, a rise of approximately 10% over a mean study period of 17 months. The rises in both systolic ($P < 0{\cdot}0005$) and diastolic ($P < 0{\cdot}0005$) blood pressures were highly significant.

Of the 21 patients who developed hypertension, four stopped treatment, following which their blood pressure returned to normal. In two patients, blood pressure returned to normal values despite continuing treatment. Six patients with raised blood pressures were treated with hypotensive agents and, in a further eight patients with mild hypertension (diastolic BP 95–100 mm Hg), no antihypertensive treatment has yet been given and treatment with CyA is being continued. One patient died from a cerebral haemorrhage.

The onset of hypertension in these 21 patients varied from 3 days to 42 months. In eight cases, hypertension occurred within 2 weeks and, in another four patients, within 4 weeks. Three patients developed hypertension within the first 12 months of therapy and, in a further six patients, after 12 months.

There was a significant difference ($P < 0{\cdot}05$) in the incidence of hypertension between those patients taking 3 mg/kg/day or less of CyA (8/23) and those taking more than 3 mg/kg/day (13/21).

Renal function. Two tests have been used to monitor renal function during CyA treatment.

1. Serum creatinine levels. Only six (14%) of the 44 patients in this study showed a persistent rise of more than 30% in serum creatinine level. The rises occurred after 2 months ($n = 1$), 1 year ($n = 1$), 3 years ($n = 1$) and 4 years ($n = 3$). This suggests that the longer the treatment, the greater is the risk of renal impairment. The two patients who showed a rise in serum creatinine at 2 months and 1 year were both taking more than 5 mg/kg/day and, in one of these, CyA therapy has been discontinued.

2. GFR, Group I. In the 13 patients who first had their GFRs measured after a mean duration of 30 months of CyA treatment, the mean GFR was 86 ml/min. After a further 12 months of treatment, the mean GFR was 82 ml/min (not significant). However, during this latter period, two patients showed reductions in GFR of 25%; both developed hypertension.

Of the 13 patients in Group I, six had low GFR values (mean 63 ml/min). Treatment was therefore discontinued for periods ranging from 3–17 weeks; GFRs rose in all six patients to a mean of 79 ml/min ($P < 0{\cdot}05$). Treatment was then restarted and the GFR measured again after a mean period of 15 weeks. Again, the GFRs decreased in all six patients to a mean of 63 ml/min ($P < 0{\cdot}05$).

In one of these six patients (aged 66 years), the GFR rose from 50 ml/min to only 60 ml/min on discontinuation of treatment. A renal biopsy was performed and there was no evidence of nephrotoxicity. Cyclosporin A treatment has therefore continued for a further 16 months (total 50 months) and her GFR fell to 37 ml/min. On discontinuing treatment for 3 weeks, her GFR rose to 67 ml/min, demonstrating that even after 4 years of CyA treatment, the impairment of renal function was apparently reversible. This patient's mean treatment dose was 4 mg/kg/day and, in addition to the fall of GFR, she developed hypertension (160/100 mm Hg); serum creatinine levels rose by more than 30% compared with the baseline value. When treatment was discontinued, the blood pressure fell to 150/90 mm Hg and serum creatinine levels were also reduced. Thus, these findings indicate that the effect of CyA on blood pressure was also reversible after long-term treatment.

3. GFR, Group 2. In the 28 patients whose GFRs were measured before and at the end of the study period (mean 8 months), there was a decrease from a mean of 108 ml/min to 91 ml/min, a reduction of 16% ($P < 0{\cdot}0001$). In six of these patients, there was a reduction of more than 25%, and five of these patients also developed hypertension. Sixteen of these patients had taken a mean CyA dose of 3 mg/kg/day or less and, in these patients, the percentage reduction in GFR was 9%. In 12 patients who had taken a mean dose of more than 3 mg/kg/day, the percentage reduction of GFR was 23% ($P < 0{\cdot}005$).

Of these Group 2 patients, 10 developed hypertension and four were hypertensive before starting CyA treatment. The mean decrease in GFR in these 14 hypertensive patients was 27% compared with the 6% mean decrease ($P < 0{\cdot}01$) in those who remained normotensive.

Malignancies. Three of the 44 patients in this study developed malignancies. One patient developed a squamous cell carcinoma of the anal margin at 6 months of treatment. After 2 years of further treatment, the patient developed two basal cell carcinomas, one on the back and another on a leg, and another squamous cell carcinoma on a leg. All four lesions, which were treated surgically, did not recur and CyA treatment was continued. After 3 years of treatment, the patient died from a cerebral haemorrhage.

The second patient was found to have carcinoma *in situ* of the cervix after 3 years of treatment. The lesion was treated by cone biopsy and there has been no evidence of a recurrence. The patient has now completed 4 years of CyA treatment.

The third patient developed a squamous cell carcinoma on the leg after 1 year of CyA treatment. She had had a similar lesion excised from her other leg 7 years earlier during PUVA therapy. Cyclosporin A treatment has been continued for 6 months so far.

Liver function. Twelve of the 44 patients had abnormal liver function tests (all mild) prior to starting CyA treatment. In nine patients, the tests have remained abnormal during treatment, but have not deteriorated; in the remaining three, the tests have become normal. Five patients, whose liver function tests were normal before treatment, developed slight abnormalities during therapy.

Haematological parameters

No abnormalities in these parameters were observed during treatment with CyA.

Cyclosporin A blood levels. Of the 13 patients whose CyA blood levels were assessed using the polyclonal antibody assay, the mean value was 498 ng/ml. Two patients had mean blood levels greater than 800 ng/ml, and both had mean blood levels above 400 ng/ml using the monoclonal antibody assay. Both had previously received methotrexate, and one of them was the youngest patient with normal liver function tests.

The mean CyA blood level for all 44 patients in this study using the monoclonal antibody test was 207 ng/ml. Four patients had mean blood levels greater than 400 ng/ml, which is considered toxic. Three of these patients had previously received methotrexate and the fourth was consuming excessive quantities of alcohol. Thus, impaired liver function may have played a role in causing these high levels. All four of these patients developed hypertension. Treatment was discontinued in two of these patients and another was the patient who died.

Cyclosporin A blood levels and hypertension

Patients who developed hypertension had a mean CyA blood level of 258 ng/ml compared with 143 ng/ml ($P < 0.01$) in those who remained normotensive.

Correlation between dose and blood levels

There was a good correlation between these two variables. In those patients receiving 3 mg/kg/day or less, the mean blood level was 143 ng/ml whereas, in those taking more than 3 mg/kg/day, the mean blood level was 296 ng/ml ($P < 0·01$).

DISCUSSION

This study has shown that a relatively low dose of CyA can produce a substantial improvement in over 90% of patients with severe persistent psoriasis. The drug appears to clear the psoriasis of all patients but, if the dose required is greater than 6 mg/kg/day, hypertension and renal impairment (that is, a ± 50% reduction in the GFR) are likely to occur. In addition, higher doses of CyA (over 6 mg/kg/day) are more likely to induce lymphomas than are lower doses (below 6 mg/kg/day).[8]

Another finding of this study is that, in over 50% of patients, a satisfactory clinical improvement can be maintained with 3 mg/kg/day or less. The question arises as to whether it is justifiable to continue treatment with CyA if hypertension and impairment of renal function, as judged by a reduced GFR, occurs. At present, there is no long-term treatment for severe psoriasis which is not without some risk. The advantage of CyA is that the risks of hypertension and nephrotoxicity can be monitored and the drug discontinued if they are severe. It is reassuring that even in patients who have been taking CyA for periods of 2–4 years, hypertension and low GFRs revert towards normal values on stopping the drug. If possible, measuring GFRs rather than relying on a greater than 30% rise in serum creatinine is a better indicator of renal function. A number of patients have shown abnormally low GFRs without a significant rise in serum creatinine levels.

The long-term risk of malignancy with low-dose CyA has still to be evaluated in patients with psoriasis. In transplantation studies, the risk of developing malignancies with this drug is no greater than with other drugs used for immunosuppression.[9] The risk of malignancy increases with time and, thus, careful monitoring of patients is mandatory. Only three of the 44 patients in this study have developed malignancies, and the one patient who developed four skin neoplasms had received long courses of PUVA and methotrexate before the CyA treatment, both of which may have been contributory.

In conclusion, long-term CyA treatment has been shown to be effective in maintaining control of psoriasis in the majority of patients. However, doses greater than 5 mg/kg/day are not recommended for long-term treatment. As expected, hypertension and nephrotoxicity were the most common side-effects. If significant rises in blood pressure cannot be controlled or if the GFR shows a progressive decrease below the lower limit of normal, then treatment should be discontinued.

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Efficacy of cyclosporin A in psoriasis: a summary of the United States' experience

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SUMMARY

Since its discovery in 1972, cyclosporin A (CyA) has been widely used in the experimental treatment of multiple inflammatory diseases considered to be of immune-mediated aetiology. In dermatology, oral CyA is most effective in the treatment of psoriasis and has been used successfully for plaque-type, pustular and erythrodermic forms of the disease. While dosages ranging from 1 to 14 mg/kg/day have been used, a starting dose of 4 mg/kg/day gives a rapid response with few side-effects. Nephrotoxicity remains the greatest concern in long-term use of the drug. Although intralesional CyA has proven effective in psoriasis, topical preparations have not. It is hoped that future research will provide effective topical formulations of CyA which are efficacious without the risks inherent in systemic administration.

Cyclosporin A (CyA) is a cyclic polypeptide of fungal origin that was discovered in 1972. Due to its marked immunosuppressive effects and lack of myelotoxicity, the drug rapidly became the mainstay of therapy for organ transplantation. In recent years, the drug has been used in the experimental treatment of rheumatoid and psoriatic arthritis, insulin-dependent diabetes mellitus, inflammatory bowel diseases, myasthenia gravis, Grave's disease and uveitis.[1]

Cyclosporin A has been used as the sole therapy for psoriasis,[2-6] alopecia areata,[7-10] Behçet's disease,[11,12] atopic dermatitis,[13,14] pyoderma gangrenosum[15-17] and lichen planus.[18-20] In combination therapy with other immunosuppressive drugs, CyA has been shown to have a beneficial effect in the treatment of dermatomyositis/polymyositis,[21-23] systemic lupus erythematosus,[24-26] pemphigus and pemphigoid.[27,28]

In dermatology, the most dramatic effect of treatment with CyA is seen in psoriasis. Mueller and Herrmann[29] made the fortuitous discovery in 1979 that plaque-type psoriasis cleared in all four patients who received the drug for psoriatic arthritis. Van Hooff *et al.* [30] reported a renal transplant recipient whose psoriasis cleared after initiation of CyA treatment.

The first controlled study to evaluate the efficacy of CyA in psoriasis was done by us in 1986.[3] Twenty-one patients were enrolled in a double-blind cross-over study. All patients had severe

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plaque-type psoriasis which had been recalcitrant to treatment with topical corticosteroids, anthralin (dithranol) and tar as well as to ultraviolet B (UVB), oral psoralen and ultraviolet A (PUVA), or methotrexate. Patients received either placebo or CyA at a dose of 14 mg/kg/day. Within 4 weeks of therapy, 20 of 21 patients given the drug showed significant to complete clearing of psoriasis. The patients who received placebo showed no response prior to cross-over to active drug treatment.

In an open study, Wentzell *et al.*[31] noted marked improvement to complete clearance in 14 patients given CyA at 5–15 mg/kg/day for 1 month. These excellent responses were seen in patients regardless of whether they had pustular, erythrodermic or plaque-type psoriasis.

Multiple studies conducted both in Europe and in the U.S. have now shown that low-dose CyA can induce clearing of psoriasis. Marks[32] treated nine patients who had plaque-type psoriasis with a starting dose of 1 mg/kg/day of CyA, increasing the dose every 2 weeks as necessary, until improvement was noted or up to a maximum dose of 5 mg/kg/day. Within 20 weeks, all nine patients showed improvement, and clearance was achieved in seven. The average dose required for a clinical response was 3·3 mg/kg/day.

Griffiths *et al.*[4] treated 10 patients with CyA at 2–4 mg/kg/day for 3 months and concluded that the lowest effective dose of the drug was 3 mg/kg/day. Fry *et al.*[33] found that they were able to maintain near-complete remission of psoriasis in eight patients, who were followed for up to 66 weeks, using CyA dosages of 1–4 mg/kg/day. Meinardi and Bos[34] induced remissions of psoriasis in 12 patients with a mean dose of 6 mg/kg/day. On reducing the dose of the drug, each patient had a dose level of CyA below which a flare of psoriasis was inevitable. This dose threshold ranged from 1·6–7 mg/kg/day which, for a given patient, varied with time. Picascia *et al.*[35] used a starting dose of CyA of 7·5 mg/kg/day to treat three patients whose plaque-type psoriasis had failed to respond to other systemic therapies. Two of these patients cleared completely within 3 weeks and one was cleared by 8 weeks. One patient has been maintained on 3·5 mg/kg/day while the other two have been successfully switched to PUVA therapy or etretinate and UVB while maintaining good control of their disease.

We conducted a dose-finding double-blind placebo-controlled trial using CyA in the treatment of severe recalcitrant psoriasis.[36] Eighty-five patients were enrolled in the first phase of this study, during which patients received either placebo, 3 mg, 5 mg or 7·5 mg/kg/day of the drug. Within 1 month of therapy, the percent improvement from baseline in each group was 0%, 42%, 61% and 79%, respectively, for each dose group. By Week 8 of therapy, the percentage improvement was −5%, 54%, 82% and 93%, respectively. In order to achieve a greater than 90% clearance by Week 16, 60% of patients taking 3 mg/kg/day required an increase in dosage compared with 25% of those in the 5 mg/kg/day dosage group and 13% of those receiving 7·5 mg/kg/day. Dosage reduction for side-effects or signs of toxicity was necessary in 0%, 0%, 5% and 33% of each group respectively. In general, the drug was very well tolerated and side-effects were similar to those reported in others taking CyA.

We suggest that CyA at 4 mg/kg/day may be the best starting dose as this can be expected to produce a significant improvement within 1–2 months of therapy with minimal risk of short-term toxicity. If an increase in dosage is required, most patients can be expected to tolerate 5 mg/kg/day, at least for several weeks to months.

Cyclosporin A has also been shown to be effective in non-plaque-type psoriasis. Picascia *et al.*[5] treated two patients who had erythrodermic psoriasis with CyA at approximately 8 mg/kg/day; complete clearance was noted in both patients within 3 weeks. After clearance was achieved, one patient was maintained on CyA 2 mg/kg/day and had plaque-type psoriasis on less than 20% of her body. The second patient was controlled with PUVA therapy and CyA at 1·7 mg/kg/day.[35] Meinardi *et al.*[37] reported successfully treating a patient with generalized von Zumbusch pustular psoriasis using CyA at 5–12 mg/kg/day. We treated one patient, who had a 30-year intermittent history of pustular psoriasis, with CyA at a starting dose of 7·5 mg/kg/day.[38] Due to his excellent clinical response, we were able to gradually taper his dose to 1·5 mg/kg/day over 5 months. A mild flare of disease at this dose required a gradual dosage increase of 3·5 mg/kg/day, which again resulted in a remission.

A recalcitrant case of acrodermatitis continua cleared for the first time in 10 years after therapy was initiated with CyA at 14 mg/kg/day, followed by gradual tapering to a dose of 5 mg/kg/day.[39] Six patients with palmoplantar pustulosis showed a dramatic improvement with CyA at 2·5 mg/kg/day.[40] We treated two patients who had acrodermatitis continua with CyA at 6 mg/kg/day.[41] One patient cleared completely and the other showed no new pustulation within 8 weeks of initiating therapy.

Intralesional CyA can be effective in the treatment of plaque-type psoriasis. Ho *et al.*[42] conducted a double-blind trial of six patients with plaque-type psoriasis which compared intralesional injections of CyA (17 mg/ml), vehicle and saline which were given three times per week. Within 4 weeks, significant improvement to complete clearing was seen in all CyA-treated plaques in contrast to vehicle- or saline-treated plaques, which showed minimal to no improvement. Both the solution of CyA and its vehicle caused pain on injection in all the patients, apparently as the result of the vehicle rather than the drug itself. In contrast, CyA administered once weekly was no better than its vehicle in improving the plaques of psoriasis. Systemic absorption of the drug was negligible, suggesting that CyA need not be given systemically to be effective in the treatment of psoriasis. Similar results were also found in a study of 10 patients.[43]

In contrast, topical CyA has, so far, been ineffective in the treatment of psoriasis. In a placebo-controlled study, Griffiths *et al.*[44] treated six patients twice daily for 4 weeks with a 2% CyA ointment. No difference was noted in efficacy between the drug and placebo. In an open trial, we treated 10 patients with a 10% CyA solution, which was also found to be ineffective,[45] and a similar lack of response was noted in six patients treated with a 5% topical CyA solution.[46] Bousema *et al.*[47] found 10% topical CyA gel to be ineffective.

Schulze *et al.*[48] treated six patients in a placebo-controlled trial using a 5% CyA ointment, under plastic-film occlusion, for 2–3 weeks. Immunostaining revealed a marked decrease in the number of infiltrating neutrophils in the epidermis and papillary dermis, but no change in the helper/suppressor T-cell ratio. Despite drug levels in CyA-treated plaques comparable to those observed in patients given oral CyA, no change in epidermal cell kinetics was noted after topical application of the drug, and no clinical improvement was seen. This is in contrast to our study of intralesional CyA in which a clinical response was preceded, in many cases, by a significant reduction in the number of antigen-presenting cells, epidermal and dermal monocytes, and keratinocyte intercellular adhesion molecule-1 expression.[42]

It is not known why topical CyA formulations have been ineffective in the treatment of psoriasis. The clearing in response to intralesional CyA demonstrates that systemic administration should not be required for efficacy. The failure of psoriasis to improve in response to topical applications of the drug may be due to an inadequate concentration of the drug reaching the necessary site(s), poor percutaneous absorption,[49] rapid redistribution of the drug, drug inactivation or by non-specific binding to proteins in the skin.

The discovery of CyA in 1972 and the subsequent demonstration of its marked efficacy in psoriasis have ushered in a new era of both research and therapy for this chronic disease. The dramatic effects of CyA in many dermatological diseases has not only provided new therapeutic options, but has also expanded our understanding of the pathophysiology of many diseases and opened up new avenues of research. It is hoped that, in the future, effective CyA topical formulations or oral analogues with less toxicity will be discovered.

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Oral cyclosporin A in the treatment of psoriasis: an overview of studies performed in The Netherlands

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SUMMARY

This is a review of the clinical studies performed so far in The Netherlands of the treatment of psoriasis with cyclosporin A (CyA), a selective immunosuppressive drug that has caused a major breakthrough in transplant medicine. Data derived from a double-blind placebo-controlled study (5 mg/kg/day of CyA), dose-finding studies (2·5 mg *vs* 5 mg/kg/day and 1 mg, 2 mg or 3 mg/kg/day), and long-term treatment of chronic plaque-type psoriasis (1·1–7·2 mg/kg/day) suggest an initial starting dose of 3 mg/kg/day irrespective of the severity of the disease. Long-term treatment brought about dose- and time-dependent (reversible) side-effects, including renal dysfunction and hypertension. Efforts to reduce the dose included concomitant administration of drugs known to have anti-psoriatic efficacy. Only combination with topical steroids appeared to add to the clinical efficacy of CyA, but did not allow a dose reduction sufficient to restore renal function. Dose reduction through intermittent treatment, however, postponed exacerbations sufficiently to permit at least partial normalization of serum creatinine levels. A similar effect was seen in the treatment of pustular palmoplantar psoriasis, which responded to doses of 1·1–6·1 mg/kg/day.

On the basis of the results of cyclosporin A (CyA) in clearing the skin lesions of psoriasis,[1-4] a systematic evaluation of the drug was started in 1985. Because of the dose-dependent side-effects of nephrotoxicity and hypertension, and the possibility of developing tumours, treatment with CyA was initially restricted to the more severe forms of psoriasis. Unfortunately, as topical CyA preparations appeared to be ineffectual in the treatment of psoriatic skin lesions, studies of alternative treatment regimes using reduced doses of CyA were carried out. Furthermore, patients were selected to determine whether the less severe forms of psoriasis required doses sufficiently low to avoid the side-effects of severe hypertension and renal

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dysfuction. These studies showed that the dosage necessary to induce and maintain a remission did not correlate with the extent of the disease.

TREATMENT OF CHRONIC PLAQUE PSORIASIS WITH CYCLOSPORIN A

In 1985, the first five patients with severe chronic plaque-type psoriasis were treated in Rotterdam in an open prospective study with 5 mg/kg/day (range 3·6–7·4 mg) of CyA for 8 weeks.[5] An almost complete remission was obtained in three patients. Three other patients showed a substantial improvement in their nails and one patient had a striking improvement in the symptoms of severe psoriatic arthropathy. A mild and reversible hypertension was observed in two patients and, in one of these, there was evidence of mild renal dysfuction. No other important side-effects were observed.

Based on these findings, a double-blind cross-over, placebo-controlled study was carried out in Rotterdam and Amsterdam[6] which included 20 patients with severe chronic plaque psoriasis. Phase I of this study comprised a double-blind comparison of 4 weeks of CyA treatment *vs* placebo, followed by a cross-over period during which those previously taking placebo received CyA for a further 4 weeks.

As two of the placebo-treated patients dropped out of the study, a total of 18 patients were evaluated. All 18 had received a mean dose of 5·6 mg/kg/day of CyA for 4 weeks (Phase I). Nearly complete remission was seen in four (22%) of these patients at the end of this period, and a reduction of more than 75% in the Psoriasis Area and Severity Index (PASI)[7] score was observed in 15 (83%).

In the following 8 weeks (Phase II), the dose required to maintain a remission was 3 mg/kg/day on average. Side-effects included mild hypertension in five patients, aching muscles in five, fatigue in two, mild hypertrichosis in two males, headache in one, mild gynaecomasty in one male, tremor in one, gastrointestinal complaints in one and verruca vulgaris in one. Subsequent withdrawal of treatment (Phase III) resulted in a relapse in all but one of the patients within 4–12 weeks of stopping the drug.

This study, while establishing the efficacy of low-dose CyA in severe forms of psoriasis, also showed that this and the side-effects were dose-dependent (Phase II) and that, in these patients, the skin lesions would recur soon after the drug was stopped (Phase III). A further argument for establishing the lowest effective dose of CyA is to avoid immunosuppression that may facilitate an accelerated expression of tumours induced by other treatments, such as ultraviolet light (UVB), psoralen photochemotherapy with UVA (PUVA), methotrexate or arsenic.

Maintenance studies

In a study[8] to establish the dose required to maintain long-term remission in severe forms of psoriasis, 12 patients were selected who had extensive but stable chronic plaque psoriasis. All the patients in the study were treated with an initial dose of 5 mg/kg/day of CyA, which was gradually reduced according to the clinical efficacy. The doses required to maintain a remission ranged from 1·1–7·2 mg/kg/day (mean 5 mg/kg/day). Improvement or worsening of the skin lesions was seen as early as the third day. The closer the dose was to the minimum maintenance dose, the slower were the changes achieved.

During maintenance treatment, six of the patients developed mild hypertension within an average of 20 weeks (range 4–49 weeks), and seven patients showed an increase in the serum creatinine of greater than 30% over their baseline values within an average of 16 weeks (range

3–22 weeks). To reverse these adverse effects, the dose was reduced but, in three patients, the dose was insufficient to control their psoriasis. In these patients, the effective long-term suppression of skin lesions was not feasible without inducing serious adverse effects.

An attempt to reduce the maintenance dose of CyA involved these 12 patients and an additional group of 14 similarly psoriatic patients who had received identical treatment with CyA. Two different treatment strategies were used. The first involved the addition of another anti-psoriatic treatment and the second was intermittent CyA treatment used as monotherapy.

In the first treatment strategy, oral CyA was combined with either UVB, anthralin (dithranol), potent topical corticosteroids or etretinate. The corticosteroids proved to be of value, but only to a limited extent, and these combinations were not able to prevent an exacerbation of the psoriasis nor allow a reduction of dosage sufficient to reverse either hypertension or renal dysfunction. No improvement of clinical efficacy was seen with a combination of CyA and bromocriptine nor with domperidone, although this latter agent was able to reduce gastrointestinal discomfort. The lack of additional effectiveness with the combination of CyA and etretinate was confirmed by Heule *et al.*[9]

Side-effects arising during a mean maintenance treatment period of 19·5 months (range 7–37 months) were assessed as a function of dose, and hypertension was observed in 2/5 patients treated with doses below 2 mg/kg/day, in 1/10 treated with 2–3 mg/kg/day, and in 5/11 treated with doses over 3 mg/kg/day. Renal dysfunction was observed in 2/5 patients receiving doses below 2 mg/kg/day, in 7/10 receiving 2–3 mg/kg/day and in 9/11 receiving doses over 3 mg/kg/day. In addition to being dose-dependent, the side-effects also appeared to be related to time. Other adverse effects included hyperkalaemia (12/26), tremor, myalgia (5/26), hypertrichosis (4/26), gastrointestinal upset, fatigue, headache and severe gingival hyperplasia (1/26).

Intermittent treatment schedule

When daily maintenance treatment was interspersed with treatment three times a week (using the same dosage as for daily treatment), reversal of CyA-related side-effects was observed and sufficient efficacy maintained for 3–20 weeks (mean 7 weeks). After that time, to avoid a serious recurrence of disease, patients returned to a daily treatment schedule for a few weeks.

The thrice-weekly treatment was also used in six patients in an attempt to induce remission (using doses ranging from 5 to 8·5 mg/kg/day of treatment). Within 6 weeks on average, PASI scores showed 85% reductions. Remission was maintained without adverse effects during the 48 weeks of the study.[8]

Nordic multicentre dose-finding study

The Nordic multicentre, open, uncontrolled study was begun in 1987 to test the efficacy, safety and tolerability of CyA in the induction and maintenance of remission in severe chronic plaque psoriasis. The study was divided into three phases: (I) induction of remission; (II) maintenance of remission; (III) reversibility of CyA-induced side-effects after withdrawal of the drug. The PASI scores had to be at least 18.

The starting dose of CyA was 2·5 mg/kg/day which, for the non-responders (reduction of PASI score $\leqslant 10\%$), was raised to 5 mg/kg/day at Week 4 or Week 8 of treatment. At Week 12, patients were classified as responders if reduction in PASI score was $\geqslant 75\%$. Patients still not responding were re-entered into the study with a starting dose of CyA 5 mg/kg/day while responders continued taking 2·5 mg/kg/day until the end of Month 12, unless there was a relapse. Patients receiving 5 mg/kg/day were 'weaned' through a gradual reduction in dosage to 2·5 mg/kg/day by the end of Month 6. During Months 13–15, all patients were weaned from CyA. Patients responding to 2·5 mg/kg/day had a mean PASI score reduction of 60%, while those receiving 5 mg/kg/day had a mean decrease of 80%.

The Multicentre 1-2-3 Study

The Multicentre Study OL 8021 (the so-called 1-2-3 Study) was designed to investigate CyA requirements in patients with mild-to-moderate psoriasis. All were resistant to topical treatment and would have otherwise been treated with PUVA/UVB or retinoids. The OL 8021 was an open prospective study in which patients were treated for 6 months with oral CyA followed by a treatment-free period of 3 months. The study included 106 patients of both sexes with chronic plaque psoriasis and all were started on oral CyA at a dose of 1 mg/kg body weight/day. If, by the end of the first month of treatment, clinical improvement was insufficient, the dose was increased to 2 mg/kg/day. If improvement was then still considered insufficient, the dose was increased to the maximum of 3 mg/kg/day. If no satisfactory anti-psoriatic efficacy was obtained with 3 mg/kg/day, the patient was withdrawn from the study.

After an active-treatment period of 6 months, therapy was stopped and patients observed for a further 3 months or until skin lesions became intolerable. The clinical efficacy of the CyA treatment was measured using the PASI score and an additional 8-point scale of improvement in skin lesions over the baseline condition. Using this scale, a treatment success was defined as a marked improvement (2/8) or total clearing (1/8) of the skin lesions; otherwise, the clinical response was considered insufficient.

Five of the 106 patients were withdrawn from the study because of a lack of clinical improvement. Many patients reported that considerable motivation was required to continue taking CyA according to the protocol.

Although the results are preliminary, in this group of patients the amount of CyA required was somewhat lower than that required for the more severe forms of psoriasis. In spite of this, approximately 70% of these patients required 3 mg/kg/day, and approximately 25% required 2 mg/kg/day to induce and maintain remission. As measured by the PASI, the 1 mg, 2 mg and 3 mg/kg/day groups did not differ in either initial severity of skin lesions or in the clinical improvement obtained with CyA treatment, a clear indication that the required amount of drug cannot be predicted by the PASI score. As treatment with 3 mg/kg/day was not known to be more likely to induce adverse effects than 2 mg/kg/day, this study suggests that treatment may start with 3 mg/kg/day of CyA, regardless of the severity of the psoriasis.

TREATMENT OF PUSTULAR PSORIASIS WITH CYCLOSPORIN A

In the trials mentioned so far, pustular forms of psoriasis have been excluded. Although CyA does not appear to have a direct effect on neutrophils, there is the possibility that, through the complex cytokine-mediated interaction between T cells, keratinocytes and endothelial cells, it may indirectly suppress pustulation.

Indeed, in a patient with von Zumbusch generalized pustular psoriasis,[10] high doses of the drug (12 mg/kg/day) suppressed pustulation although, in two other similar cases,[11,12] lower doses (7 mg and 8 mg/kg/day) were ineffective. However, when prednisone 10 mg/day was added to the CyA 8 mg/kg/day treatment, the skin lesions were completely cleared. Such high dosages of CyA inevitably produce side-effects such as hypertension and renal dysfunction, especially when the treatment is long-term. In pustular psoriasis, therefore, CyA should preferably be reserved for only those patients not responding to etretinate alone or with PUVA, or for cases where hepatotoxic drugs are contraindicated.

In a prospective open study of the clinical efficacy and tolerability of CyA, seven patients with persistent pustulosis palmaris et plantaris (PPP) were treated with oral CyA.[13] Clinical efficacy was assessed on a semi-quantitative 0–4-point scale for erythema, desquamation, induration and pustulation. Long-term CyA treatment (mean 19 months, range 5–38 months) was effective at doses ranging from 1·1–6·1 mg/kg body weight/day. Side-effects included renal impairment (1/7, controlled by a slight reduction of the dose), nausea (3/7), hypertrichosis (2/7) and tiredness (3/7). Rapid recurrence of the skin lesions was observed on withdrawal of the drug or when the dose was insufficient.

RECOMMENDATIONS FOR TREATMENT

These trials have produced impressive results in the treatment of psoriasis with oral CyA. However, the risk of adverse effects, such as hypertension, renal dysfunction and the possibility of producing tumours, should discourage its prolonged use. Cyclosporin A should not be given to patients with a history of frequent treatments with PUVA, UVB, methotrexate or arsenic.

As 3 mg/kg/day of CyA does not produce more side-effects than 2 mg/kg/day, as indicated by the results of a study of mild-to-moderate psoriasis, treatment should begin at the higher dose. Treatment commencing with too low a dose would only impede improvement. After 2–4 weeks, the dose can be adjusted according to the efficacy. Severe forms of psoriasis may require substantially higher doses, as much as 7 mg/kg/day, but such high doses should be allowed only in special cases and with frequent monitoring for adverse effects. Complete clearance of the skin lesions should not be the sole objective of treatment.

Further clinical improvement may be achieved with CyA in combination with topical corticosteroids. If side-effects arise, the dose should be lowered by 0·5 mg/kg/day at intervals of 2 weeks. After withdrawal of treatment, skin lesions can be expected to recur between the third day and twelfth week.

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Efficacy of low-dose cyclosporin A in psoriasis: results of dose-finding studies

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SUMMARY

The efficacy of cyclosporin A (CyA) in severe psoriasis was analysed in 457 adult patients included in five European multicentre dose-finding studies. Initial CyA doses were 1·25 mg/kg/day in 33 patients, 2·5–3 mg/kg/day in 285 and 5 mg/kg/day in 139. After 3 months of treatment, the reduction of the Psoriasis Area and Severity Index (PASI) score was 35 ± 6% with 1·25 mg/kg/day of CyA, 57 ± 2% with 2.5 mg or 3 mg/kg/day and 86 ± 2% with 5 mg/kg/day ($P < 0.001$). The rates of success, defined by a PASI score reduction $\geqslant 75\%$ or a score $\leqslant 8$, were 24%, 52% and 88%, respectively. There were no differences in age, initial severity or duration of psoriasis. The improvement was maintained for 9 months or more in the majority of patients receiving continuous CyA therapy.

The efficacy of cyclosporin A (CyA) in the treatment of psoriasis was recognized soon after it came into use. A dramatic improvement of skin lesions was observed in four patients in a pilot study of CyA in psoriatic arthropathy.[1] This effect was later confirmed in other pilot[2-11] and controlled[12,13] studies. Of major importance was the finding that low-dose CyA (less than 5 mg/kg/day) was also effective.[3,14-16]

Because of the potential risk of renal impairment associated with CyA, it was essential to identify the lowest effective dose of CyA.[17] The relationship between dose and efficacy as well as the speed or duration of improvement was investigated in several multicentre studies initiated by Sandoz. This report presents the pooled analyses of these studies. In addition, this large series of patients provided the opportunity to investigate the influence of the patients' baseline characteristics on the clinical response.

PATIENTS AND METHODS

This analysis included 457 adult patients (mean age 42 years, range 18–75) in five European multicentre dose-finding studies. All patients had severe psoriasis, defined by a PASI score $\geqslant 18$ (see below), for a mean duration of 14 years. The mean PASI score at inclusion was 25.

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Cyclosporin A therapy

The initial CyA doses (divided into two daily doses) were 1·25 mg/kg/day in 33 patients, 2·5 mg or 3 mg/kg/day in 285, and 5 mg/kg/day in 139. Treatment lasted for 3 months in the five studies and, in two of them, the patients with clinical success after 3 months of treatment entered a maintenance period of continuous therapy for up to 12 months.

Doses were adjusted on the basis of efficacy or renal dysfunction. In cases of inefficacy after 1–3 months treatment, the dose was increased but without exceeding 5 mg/kg/day. Whenever renal dysfunction was observed, defined as a serum creatinine increase of more than 30% over patient's own baseline, the dose was reduced by 25–50%.

Patients who started on 5 mg/kg/day of CyA entered the maintenance period at that dose and began 3 months of gradual reduction of the dose to 2·5 mg/kg/day. Patients who initially had success on 2·5 mg/kg/day continued on that dose.

Evaluation of severity of psoriasis

Evaluation of the severity of the disease was assessed in all studies by the Psoriasis Area and Severity Index (PASI).[18] This is a clinical assessment of the area of the body affected and intensity of the three main lesions: erythema, desquamation and infiltration. Each symptom is scored as absent (= 0), slight (= 1), moderate (= 2) or severe (= 3). The involved area is estimated and scored within a range of 0% (score = 0) to 90–100% (score = 6). The assessments are made separately for four different areas of the body and, using a simple mathematical procedure, a single figure emerges. Thus, a PASI score may describe either very severe lesions over a relatively small area of the body, or less severe lesions but over a larger area. To reduce the variability among the assessors, special PASI training sessions were organized in which all investigators examined the same patient, compared the results and discussed the ratings if there was a disagreement. Success was defined as a PASI score reduction of 75% or more, or a score ≤ 8. A relapse was defined by an increase of 50% or more over the score at the time of remission.

In most of the patients, global assessments were made by the investigator and by the patient, using a scale of five degrees for efficacy: none, slight, moderate, good or very good.

Statistical tests

Results are reported as means ± SEM. In cases of inefficacy or failure before Month 3, the last value was carried forward (intention to treat). The statistical significance of the difference between the groups was assessed by Student's *t*-test. The frequency of abnormalities between groups was compared using Fisher's exact test.

RESULTS

Comparison of PASI changes and global assessment

A strong correlation was found between the changes of PASI score at Month 3 and the patient's or investigator's global assessment (Fig. 1). In patients who reported very good responses, the PASI score reduction was 89 ± 1%, whereas, in cases of inefficacy, the PASI score was unchanged (an increase of 1 ± 4%). Of the patients who had PASI score reductions of 75% or more, 73% had a very good result, 25% a good result and 2% a moderate improvement. Interestingly, of the patients who had PASI score reductions of 50–74% and who were

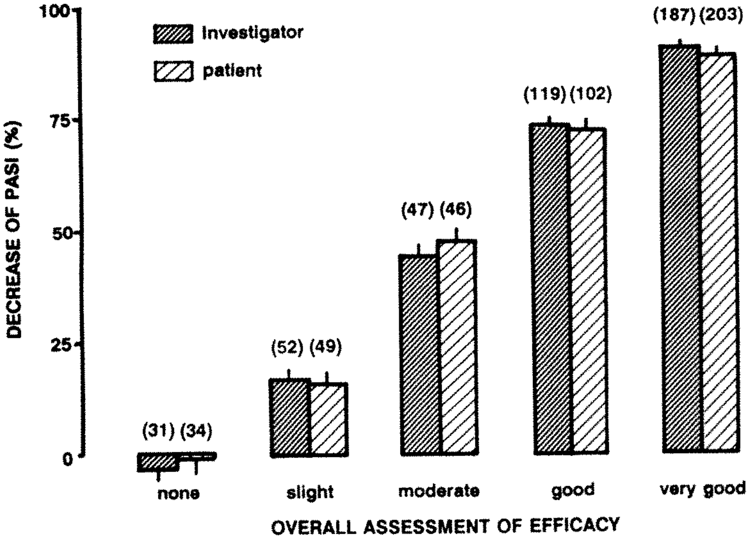

FIGURE 1. Percentage reduction of the Psoriasis Area and Severity Index (PASI) score according to the overall assessment of efficacy by the patient or the investigator after 3 months of treatment. Means ± SEM.

considered to be 'failures', the global assessment of efficacy was very good in 14% and good in 50%.

Induction of remission

The percentage of PASI score reduction after 3 months of treatment was 35 ± 6% with 1·25 mg/kg/day of CyA, 57 ± 2% with 2·5 mg or 3 mg/kg/day and 86 ± 2% with 5 mg/kg/day ($P < 0{\cdot}001$ *vs* all groups) (Fig. 2). Success rates at Month 3 were 24%, 52% and 88%, respectively ($P = 0{\cdot}001$) (Fig. 3). Patients' responses were also quicker, depending on dose, as the success rates after only 3 weeks of treatment were 6%, 10% and 35% in the 1·25 mg, 2·5 mg or 3 mg and 5 mg/kg/day CyA dose groups, respectively ($P = 0{\cdot}001$). The incidence of complete clearing of the psoriasis at 3 months was 0%, 3% and 17% for each dose group, respectively.

Seventy-one patients had their CyA doses increased after 3 months from 2·5 mg to 5 mg/kg/day because of insufficient efficacy. The success rate after 3 additional months with 5 mg/kg/day was 77%.

No significant difference in clinical response was detected according to age, gender, initial severity or duration of psoriasis with 2·5 mg (Table I) nor with 5 mg/kg/day of CyA. However, a slightly, but significantly, higher success rate was achieved in the absence of previous treatment with retinoids ($P = 0{\cdot}047$) or methotrexate ($P = 0{\cdot}001$) in the CyA 2·5 mg/kg/day group. No difference was seen with CyA at 5 mg/kg/day (Table 2).

Maintenance therapy

A subgroup of 72 patients was continuously treated with 2·5 mg/kg/day for 9 months after initial success at 3 months with that dose. Fifteen (21%) relapsed while receiving 2·5 mg/kg/day of CyA after a mean treatment duration of 6·7 months. In a subgroup of 62 patients who

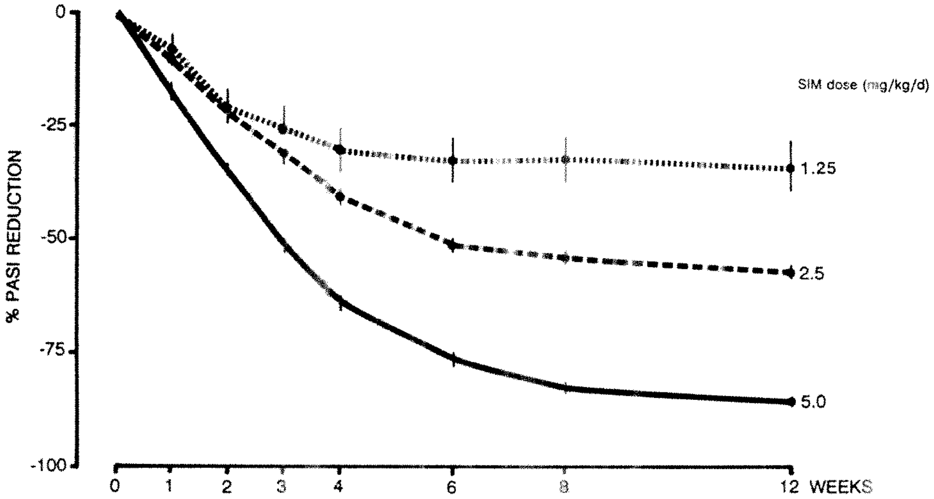

FIGURE 2. Percentage PASI score reduction according to dose in the first 3 months of treatment (intention to treat). Means ± SEM; SIM = CyA.

had success with an initial dose of 5 mg/kg/day, CyA was gradually reduced over 3 months to 2·5 mg/kg/day. Twenty (39%) of these 62 patients had a relapse at a mean dose of 3·5 mg/kg/day after a mean duration of 7 months of CyA treatment.

DISCUSSION

In the present study, the analysis of the efficacy was based on the changes of the PASI, a clinical

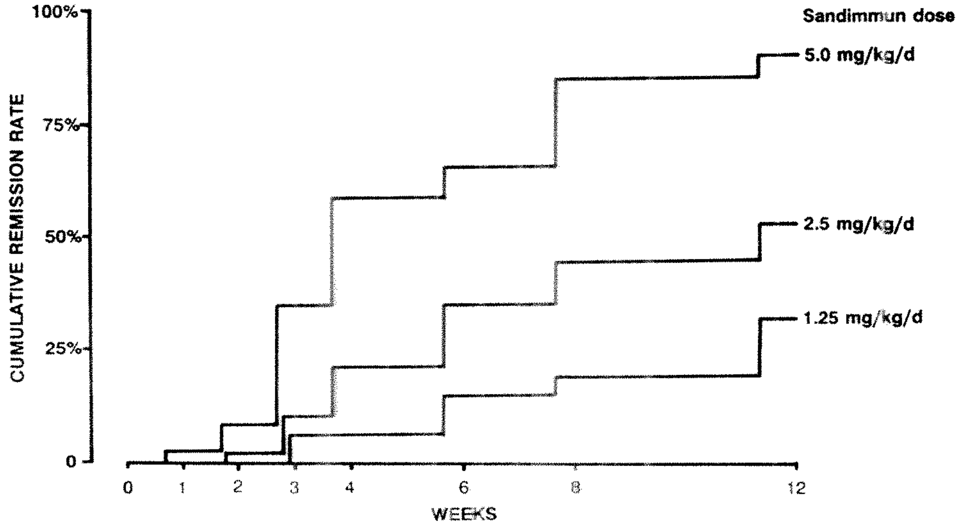

FIGURE 3. Cumulative success rate in the first 3 months of treatment according to Sandimmun® (CyA) dose. Success was defined as a PASI score reduction ≥75% or a score of ≤8.

TABLE 1. Clinical response of psoriasis with CyA (2·5 mg/kg/day) according to baseline characteristics

		Success rate n (%)	% PASI reduction
Age	≤ 40 years	51/129 (40)	55 ± 3
	> 40	57/120 (47)	60 ± 3
Gender	Male	74/173 (43)	58 ± 3
	Female	34/68 (50)	55 ± 6
PASI score	≤ 25	54/151 (36)	57 ± 3
	> 25	44/90 (49)	59 ± 4
Psoriasis duration	≤ 15 years	55/123 (45)	58 ± 3
	> 15 years	53/116 (46)	58 ± 3

Means ± SEM after first 3 months of treatment.
Success = PASI score reduction ≥ 75% or a score of ≤ 8.

score. Changes in this score correlated very well with the global evaluation of efficacy by the patient or the investigator. The PASI score reduction ≥ 75%, used in the protocols to define a success, was equivalent to a good or very good response in 98% of the patients. On the other hand, the PASI score underestimated the improvement in some patients, which is easily understandable as some particularly disabling sites, such as the face or palms, have only a limited contribution to the PASI score. Furthermore, in some cases of chronic and resistant psoriasis, a limited improvement may have been considered a very good response. Indeed, among the patients who were considered failures but who had a PASI reduction of 50–74%, the overall patients' evaluation was good to very good in 64%.

This pooled analysis confirmed the dose–response relationship with CyA doses at 1·25 mg, 2·5 mg or 3 mg, and 5 mg/kg/day which was significant after only 3 weeks of treatment. Only

TABLE 2. Clinical response of psoriasis according to previous therapy

	CyA (2·5 mg/kg/day)		CyA (5 mg/kg/day)	
	Success rate n (%)	% PASI reduction	Success rate n (%)	% PASI reduction
Methotrexate				
Yes	19/59 (32)	38 ± 5	27/31 (87)	84 ± 2
No	108/182 (59)	64 ± 2	70/81 (86)	87 ± 3
Retinoids				
Yes	62/131 (47)	53 ± 3	51/58 (88)	87 ± 2
No	65/108 (60)	63 ± 3	46/54 (85)	86 ± 3

Means ± SEM after first 3 months of treatment.
Success = PASI score reduction ≥ 75% or a score of ≤ 8.

a small number of the patients had a complete clearing of psoriasis but, as recently suggested,[19] this should not be the goal of the treatment. The response rate was quicker and higher with CyA 5 mg than with 2·5 mg/kg/day. However, with this latter dose, 52% of the patients had success by the third month. With CyA at 1·25 mg/kg/day, the improvement was very slow. The very significant response rate observed with 2·5 mg/kg/day indicates that this should be the standard starting CyA dose as it is accompanied by a low incidence of renal dysfunction.[17] In addition, 77% of the failures with 2·5 mg/kg/day showed success after increasing the CyA dose to 5 mg/kg/day. Hence, no tachyphylaxis was observed in patients who started with low doses of CyA which were increased because of insufficient efficacy.

The rate of success with CyA at 5 mg/kg/day did not differ according to the patients' baseline characteristics or previous therapies for psoriasis. However, a slightly lower rate of remission was observed with 2·5 mg/kg/day in patients previously treated with methotrexate or retinoids. These patients may have been particularly severely affected or resistant to treatment as no relationship between CyA blood trough levels and previous therapies was detected.[20]

Improvement of the psoriasis was maintained in the majority of patients taking a stable CyA dose. In the 5 mg/kg/day group, the attempt to reduce the dose to 2·5 mg/kg/day resulted in a relapse in 39% of patients. This relatively high incidence may be explained, at least in part, by the definition of relapse, which was an increase in PASI score of 50% over that at the time of success. In the patients with complete (or nearly) clearing, a minimal recurrence was considered a relapse. However, it is very likely that the maintenance dose is very close to the minimum effective dose for inducing an improvement.

It is known from the early experience with CyA in severe psoriasis[1-3] that a relapse occurs within weeks after stopping treatment, and that continuous therapy for as long as 3 years is required to maintain a response.[21,22] Thus, it is essential to remain at the lowest effective dose for a given patient which, for the majority, should be 2·5–3 mg/kg/day of CyA.

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Cyclosporin A in atopic dermatitis

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SUMMARY

Over the past 10 years, oral cyclosporin A (CyA) has been used to treat a range of inflammatory and immunologically mediated dermatological conditions, but only psoriasis has been shown to be responsive in double-blind, controlled trials. Thirteen patients with severe atopic dermatitis were treated for up to 31 months. Ten patients had a good response, one patient a moderate response and two a slight response. In two of these patients, CyA was withdrawn after more than 2 years of continuous treatment because of increasing plasma creatinine levels. Cyclosporin A appears to be useful in the treatment of severe, therapy-resistant atopic dermatitis, but careful monitoring of side-effects and dose adjustments are required.

Cyclosporin A (CyA) has been used to treat a range of inflammatory and immunologically mediated skin diseases.[1] However, double-blind placebo-controlled trials[2,3] have confirmed that only in psoriasis is orally administered CyA therapeutically effective. There is now increasing uncontrolled evidence suggesting that CyA is useful in atopic dermatitis. In 1987, Van Joost *et al.*[4] reported favourable responses in two patients given low-dose CyA for 6–8 weeks. Subsequently, Logan and Camp[5] found CyA to be helpful in two patients treated for 5 and 11 months, respectively. In 1989, Taylor *et al.*[6] described six patients with atopic dermatitis given CyA 6 mg/kg/day for 8 weeks. Five patients responded, four experiencing greater than 50% improvement within 4 weeks. One patient failed to respond and, of the initial responders, three flared during treatment. The authors concluded that CyA may be beneficial in some patients with atopic dermatitis. Two descriptions of the effectiveness of CyA in single cases of atopic dermatitis have also been reported.[7,8] The experience in Newcastle-upon-Tyne in the U.K. is that CyA is highly effective in the majority of patients who have atopic dermatitis treated with oral CyA (S. Shuster, personal communication), although such findings have not been universal (K. Wolff, personal communication).

This is a report of uncontrolled observations in 13 patients who had severe, therapeutically unresponsive, atopic dermatitis treated with CyA for 1–31 months.

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TABLE 1. Clinical data of 13 patients given CyA for severe atopic dermatitis

Patient number	Age	Gender	Treatment duration (months)	Previous treatment	Global response
1	40	F	9	SS	Good
2	64	F	9	SS, AZ, PUVA	Good
3	38	F	26	PUVA	Good
4	30	M	31	SS, AZ, PUVA	Good
5	60	M	25	SS, AZ, PUVA	Good
6	39	M	6	—	Good
7	36	M	3	SS, AZ, PUVA	Good
8	18	F	5	UVB	Slight
9	20	F	7	SS, PUVA	Slight
10	26	F	8	PUVA	Moderate
11	25	M	4	SS, PUVA	Good
12	31	M	2	—	Good
13	37	M	4	SS	Good

SS, systemic steroids; AZ, azathioprine.

PATIENTS

Over the past 4 years, 13 adult patients (six females and seven males; age range 18–64 years) have been treated with low-dose CyA (2–6 mg/kg/day). All patients fulfilled the diagnostic criteria of Hanifin and Rajka for atopic dermatitis.[9] All patients had failed to respond to conventional treatment with emollients, topical steroids and oral antihistamines, and all but one had been admitted on at least one occasion for intensive topical therapy. All were judged to have sufficiently severe disease to justify treatment with CyA. Eight patients had received at least one course of systemic corticosteroids, four had received oral azathioprine, and eight had undergone PUVA photochemotherapy (Table I). This treatment was not continued because of unsatisfactory clinical responses or, in a minority, because of the occurrence of side-effects.

With one exception, none of the patients had a history of hypertension, or renal, liver or malignant disease, and none was receiving drugs considered to interfere with the pharmacokinetics of CyA. The exception was a 65-year-old female with severe atopic dermatitis who had, 20 years previously, undergone cone biopsy followed by hysterectomy for apparent intraepithelial cervical neoplasia. Gynaecological examination had, however, revealed that she was free of genital disease.

MANAGEMENT

Cyclosporin A treatment was begun in eight patients in the outpatients' clinic and these patients were seen at 1- to 2-week intervals for 4–6 weeks. In the remaining five patients, CyA treatment was begun during a period of admission to hospital. Once stabilized, patients were seen at 4-week intervals. Starting doses of CyA varied from 2 to 5 mg/kg/day and were given in divided doses except for three patients who took once-daily doses. Maintenance doses ranged between 2·5–6 mg/kg/day (average 4 mg/kg/day) and were the lowest dosages able to maintain adequate disease control. Most patients, therefore, had some residual degree of dermatitis because of this therapeutic principle of using the lowest possible maintenance dose.

Patients were encouraged to use emollients and topical steroids, and oral antihistamines and antibiotics were prescribed when necessary.

Monitoring of side-effects was carried out at each visit and comprised blood pressure estimation, urinalysis, full plasma biochemical screening including creatinine, urea, electrolytes, uric acid, alkaline phosphatase and alanine transaminase. Creatinine clearance measurements have been performed at intervals of approximately 3 months in the last 18 months. Whole blood trough CyA levels were determined regularly at the outset, but were subsequently carried out infrequently as the dosage of CyA was determined by clinical response and monitoring of side-effects. These studies were not company-sponsored, but were funded by the National Health Service as part of normal patient care.

CLINICAL RESPONSES AND SIDE-EFFECTS

Within 1–4 weeks of starting CyA treatment, all patients showed some improvement in the extent and severity of disease and degree of pruritus. On global assessment, this improvement was judged to be good in 10 patients, moderate in one and slight in two (Table I). Nine patients responded dramatically with substantial improvement noted within 1 week of starting treatment. In the remaining four patients, improvement (although only slight in two cases) was noted after 2–4 weeks.

Whenever possible, the dose of CyA was reduced to the minimum effective level. In two patients (numbers 8 and 9 in Table I), the response to treatment was only slight in spite of an increase in CyA dose to 6 mg/kg/day. In both patients, 12-hour trough blood levels of CyA were low with this dose (54–87 ng/ml, monoclonal antibody radioimmunoassay). These observations were inexplicable as neither patient was receiving drug treatment known to interfere with the pharmacokinetics of CyA. In view of their only slight responses to treatment, CyA was stopped after 5 and 7 months in these two patients, respectively. One patient subsequently improved while receiving traditional Chinese herbal remedies, but the other was lost to follow-up.

CyA was stopped in two patients (numbers 4 and 5 in Table I) after 25 and 31 months, respectively, because of progressive increases in plasma creatinine. Plasma creatinine levels did not fall substantially following reduction of CyA dose (Figs 1 and 2), and the lower doses were associated with worsening of the dermatitis. Patient number 4 (Table I) was subsequently controlled with oral prednisolone (maximum dose 15 mg/day). The prednisolone was gradually tapered and stopped after 7 months. The patient then remained in remission without treatment other than emollients, and was discharged from follow-up. The other patient (number 5 in Table I) required re-admission to hospital for intensive topical treatment because of an erythrodermic flare. PUVA photochemotherapy was successfully reintroduced and, at the time of writing, has provided adequate control for 6 months. In both of these patients, plasma creatinine levels returned to baseline within 4–5 months of stopping CyA.

One patient (number I in Table I) stopped CyA treatment after 9 months during a period of disease quiescence while on holiday and, since then, has been able to maintain adequate disease control with topical steroids. At present, the remaining eight patients are continuing with CyA therapy. Seven have been considered to be well controlled, and one (number 10 in Table I) moderately so.

Three patients have required treatment with oral nifedipine for CyA-induced hypertension. In one case, this was changed to atenolol because of side-effects and insufficient control. Significant infection was encountered in only one patient (number 5 in Table I), who developed a low-grade

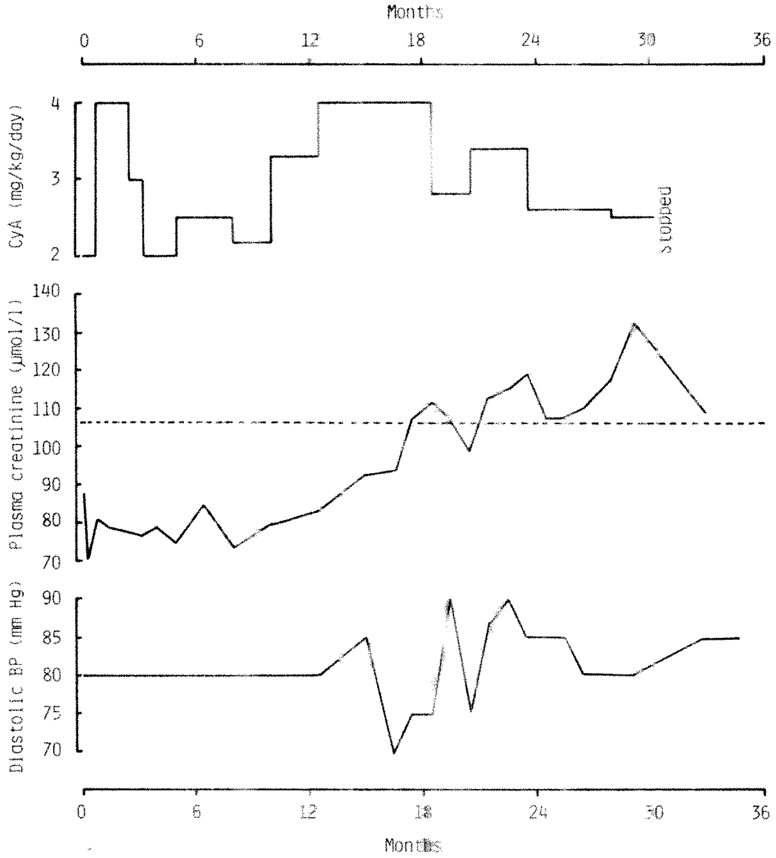

FIGURE 1. CyA dose, plasma creatinine and diastolic blood pressure levels in patient number 4 during 31 months of CyA treatment.

staphylococcal folliculitis of both forearms which was unresponsive to repeated courses of flucloxacillin. This problem was present both before and during CyA therapy, but cleared after CyA was stopped. No cases of herpes simplex infection were encountered.

COMMENT

Low-dose oral CyA appears to have been effective in inducing clinically useful improvement in 11 out of 13 patients with atopic dermatitis. These patients all had severe disease, and CyA appeared to be more beneficial than previous regimes in all but two cases. Cyclosporin A was subjectively well tolerated and, in the majority, was associated with substantial improvement within 1 week.

These results are encouraging and, in the U.K., a multicentre randomized, double-blind, placebo-controlled, cross-over study of CyA in atopic dermatitis is nearing completion. The safety of short- and long-term CyA in atopic dermatitis also requires further assessment. At present, its use should be restricted to patients with severe disease unresponsive to conventional treatment and for whom PUVA photochemotherapy is unsuccessful, contraindicated or not possible. To our knowledge, CyA has not been used in the treatment of children with severe atopic dermatitis. Careful monitoring and adjustment of dosage to the lowest effective level are

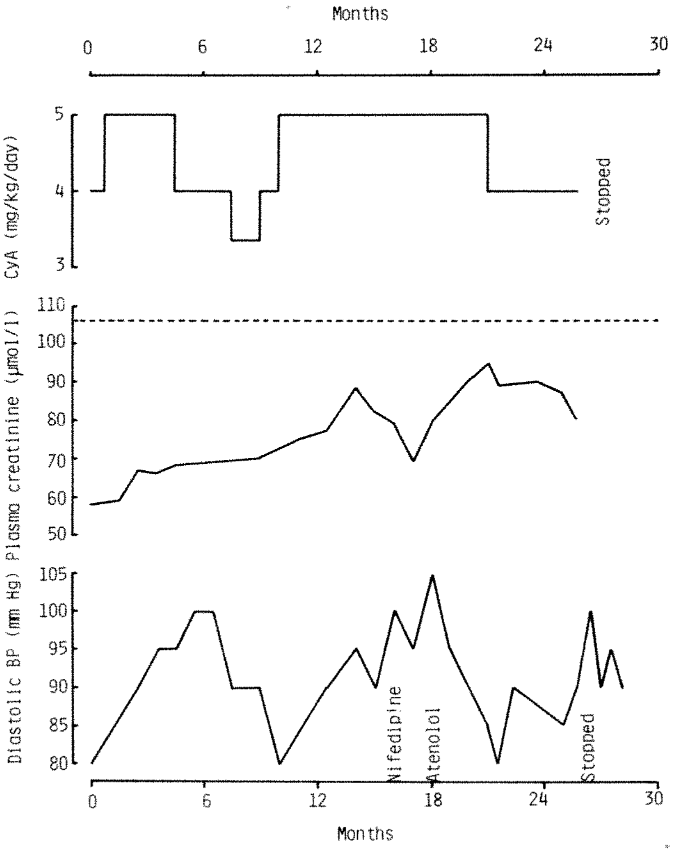

FIGURE 2. CyA dose, plasma creatinine and diastolic blood pressure levels in patient number 5 during 26 months of CyA treatment.

essential in all cases. The exclusion criteria and other guidelines outlined elsewhere in this supplement regarding the use of CyA in psoriasis should, as far as possible, be strictly followed.

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Side-effect profile of cyclosporin A in patients treated for psoriasis

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SUMMARY

This is a review of the side-effects of cyclosporin A (CyA) in patients with severe psoriasis; renal dysfunction and hypertension are discussed elsewhere. In particular, paraesthesia, hypertrichosis, gingival hyperplasia and gastrointestinal disorders may occur, but are generally transient, mild-to-moderate in severity and only rarely require discontinuation of CyA. Infections are not a problem. As expected with an immunosuppressive drug, there is the possible risk of tumour development, particularly squamous cell carcinomas. However, these skin malignancies developed almost exclusively in patients previously treated with PUVA and/or methotrexate. The few lymphoproliferative disorders regressed spontaneously on discontinuation of the drug. Whether the isolated cases of solid tumours were CyA-related is not known. Apart from a raised serum creatinine, an important indicator of renal dysfunction, the laboratory abnormalities included hypomagnesaemia, hyperkalaemia, increased uric acid, changes in liver function tests, and fluctuations in the serum cholesterol and triglyceride levels. Although most of these changes were not clinically relevant, laboratory monitoring of patients with psoriasis treated with CyA is essential.

Recent clinical investigations have shown that cyclosporin A (CyA) is effective in the treatment of severe psoriasis.[1,2] Rapid improvements have been observed with doses of 2·5–5 mg/kg/day.[3,4] This review concerns the adverse effects in a large series of patients with psoriasis treated with CyA. The data are the pooled results of therapeutic trials conducted by Sandoz Pharma Ltd. The effects of CyA treatment on renal function and blood pressure are not discussed as they are comprehensively reviewed elsewhere in this supplement.[5,6]

PATIENTS AND METHODS

The data are primarily based on 11 clinical studies assessing the effectiveness and safety of CyA in the treatment of patients with severe psoriasis. A total of 631 patients were treated with an initial daily dose ranging from 1·25–5 mg/kg/day. Only 15 patients were treated with doses of

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TABLE I. Adverse events reported* in CyA-treated psoriatic patients

Symptoms	Patients with adverse events *n*	%
Paraesthesia	49	11·1
Hypertrichosis	30	6.8
Headache	28	6·3
Gingival hyperplasia	19	4·3
Nausea	17	3·8
Fatigue	11	2·5
Abdominal pain	10	2·3
Diarrhoea	9	2·0
Dyspepsia	9	2·0
Asthenia	8	1·8
Back pain	7	1·6
Muscle cramps	7	1·6
Peripheral oedema	6	1·4
Tremor	5	1·1
Vomiting	5	1·1
Gastrointestinal complaints	5	1·1

*Incidence > 1%.

Number of patients treated = 422; duration of treatment = 12 weeks; CyA dose = 2·5–5 mg/kg/day.

Whether each of these events is CyA-related is not known.

up to 7·5 mg/kg/day. In most studies, the dose was adjusted according to the efficacy and/or the occurrence of renal or hepatic dysfunction, and the treatment period was 12 weeks. However, two studies included long-term follow-up of patients receiving maintenance treatment for up to 16 months.

RESULTS

Adverse events

Table I presents the adverse events observed in patients treated with daily doses of 2·5–5 mg/kg/day, the recommended range of CyA. However, four types of adverse events are not included: renal dysfunction and hypertension associated with CyA treatment (reviewed elsewhere); infections and malignancies (discussed separately below). Only those side-effects with an incidence greater than 1% are listed.

Most of the side-effects associated with CyA were not considered severe by the reporting physician. Only rarely did these manifestations result in discontinuation of the drug. Several adverse events, in particular headache, nausea, fatigue and gastrointestinal disorders, were also reported in the placebo group.

Paraesthesia, often described as a burning sensation of the hand, and tremor usually occurred during the first weeks of treatment and subsequently disappeared without reduction of the daily CyA dose. The aetiology of these neurological side-effects remains unknown, although hypomagnesaemia as the result of CyA-induced renal dysfunction may be responsible.[7] The cause of hypertrichosis is also unknown, and there is no evidence of any endocrine abnormality.[8,9] Likewise, the aetiology of gingival hyperplasia due to fibrous hyperplasia[10] has not been

TABLE 2. Infections reported in CyA-treated psoriatic patients

Symptoms	Patients with infections *n*	%
Skin		
Folliculitis	10	1·6
Verrucae	7	1·1
Dermatitis	5	0·8
Herpes simplex	2	0·3
Herpes zoster	1	0·2
Mycotic infection	1	0·2
Other	4	0·6
ENT and respiratory tract		
Upper respiratory tract	48	7·6
Influenza-like symptoms	45	7·1
Bronchitis	7	1·1
Pneumonia	2	0·3
Otitis media	2	0·3
Others		
Candidiasis of the mouth	1	0·2
Orchitis	1	0·2
Urinary tract infections	1	0·2

Number of patients treated = 631; duration of treatment = up to 16 months; CyA dose = 1·25–7·5 mg/kg/day. Whether these infections are CyA-related is not known.

established. Gastrointestinal disorders, including nausea, dyspepsia and diarrhoea, were frequently reported, but were seldom severe.

The drug was stopped because of side-effects in 37 out of 631 CyA-treated patients. Apart from renal dysfunction, hypertension, infections and malignancies, the reasons for discontinuing were: gastrointestinal disorders, including nausea, in three patients; hypertrichosis in two patients; back pain of unknown aetiology in two patients; and a transient ischaemic attack in two patients. Of the latter two, one presented with severe arteriosclerotic plaques and, in the other, the cause of the ischaemic incident was not established.

In a further six patients, discontinuation of treatment was due to myocardial infarction, acute cholecystitis, jaundice associated with renal dysfunction, scotoma, monoclonal immunoglobulin peak, and paraesthesia developing at 1, 2, 4, 8, 13 and 15 weeks, respectively, after starting treatment. Except for the paraesthesia, the causal relationships of these single events remain questionable.

Infections

These are not a particular problem in CyA-treated patients with psoriasis, and their reported incidence is shown in Table 2. Skin infections, such as folliculitis, are known to occur more frequently in patients with psoriasis, and infections involving the upper respiratory tract or producing influenza-like symptoms occurred primarily during the winter months. Only three patients discontinued the drug because of an infection.

The controlled studies showed no difference between CyA-treated patients and those receiving placebo or etretinate in the frequency of infections. In all cases, if spontaneous recovery did not occur, stopping the drug and/or appropriate management led to rapid resolution of the infection.

Malignancies

A total of 842 patients with psoriasis received CyA in the Sandoz Pharma clinical study programme; six patients developed malignant or pre-malignant skin lesions (three squamous cell carcinomas, one squamous and basal cell carcinoma, one basal cell carcinoma and one Bowen's disease). Solid organ tumours were observed in six further patients, namely, carcinomas of the bladder, cervix, colon, thyroid and oral mucosa, and a lymph node metastasis of an unknown primary tumour. In addition, five patients developed lymphoproliferative disorders; three were histologically benign and limited to the skin, one was a B-cell lymphoma and one a cutaneous T-cell lymphoma.

Four other patients who also developed malignancies (three squamous cell carcinomas of the skin, one breast cancer) were being treated in clinics independent of the Sandoz Pharma clinical study programme.

These malignancies were considered unlikely to be CyA-related by the investigator or reporting physician. The details of these patients are given in Tables 3 and 4. The mean ages of the patients who developed skin and solid organ tumours and lymphoproliferative disorders were 51·8 and 50·8 years, respectively. The latter were detected after an average of 3·3 months (range 0·5–6 months), and the skin and other tumours developed after an average of 11·2 months (range 2–30 months).

The crude incidence rates were 0·7% for both skin cancer and solid organ tumours, and 0·2% for lymphomas, based only on the results of patients in the Sandoz Pharma clinical study programme. However, to calculate the true incidence, the number of patients taking the drug is required and this is not known for treatment outside of the Sandoz programme. All patients were included in the calculations regardless of whether the event was CyA-related so that these figures are significantly higher than they would otherwise be.

Almost all of the patients who developed skin cancer had previously received treatment with other known carcinogens, including PUVA, UVB or methotrexate, and the skin tumours may have developed independently. The skin cancers were not aggressive and surgical excision was curative. The majority of the lesions were small and may only have been detected because of the careful and intensive examinations of the skin by the specialists involved in these trials.

The three cases of benign cutaneous lymphoproliferative disorders and the B-cell lymphoma regressed rapidly on discontinuation of CyA. The latter was polyclonal, and its rapid reversibility in this early state is well known from CyA-treated transplant patients.[11,12] The relationship of the T-cell lymphoma (mycosis fungoides) to CyA treatment was questionable as this type of malignancy also developed in a patient receiving placebo. The investigators involved with these cases suggested that early lesions may have been present on entry to the study, but were unrecognized. Whenever atypical lesions are seen, whether before or during CyA treatment, a biopsy of the lesion should be performed.

Laboratory abnormalities

The laboratory abnormalities found in patients during CyA treatment are summarized in Table 5. Except for serum creatinine—discussed elsewhere in this supplement[5]—only the abnormal laboratory parameters are included. All data were obtained during the first 12 weeks

TABLE 3. Malignancies* arising with CyA treatment of 842 patients with psoriasis participating in Sandoz Pharma clinical study programme

Type	n	Gender	Age (years)	Previous therapy	Duration of CyA treatment (months)	Discontinuation	Other treatment	Metastasis/ outcome
Skin tumours								
Squamous cell carcinoma	6[†]	5M 1F	45–63	PUVA/MTX ± etretinate	6–16	Yes	Surgery	Recovered
Squamous + basal cell carcinoma	1	M	77	UVB/MTX, retinoids	6	No	Surgery	Recovered
Basal cell carcinoma	1	M	Unknown	Unknown	2	No	Surgery	Recovered
Bowen's disease	1	M	33	PUVA/MTX	3	No	Laser therapy	Recovered
Solid tumours								
Squamous cell carcinoma of the mouth	1	M	59	Unknown	3	Yes	Surgery	Recovered
Breast carcinoma	1[‡]	F	62	—	< 3	Yes	Surgery, tamoxifen	Recovered
Cervix carcinoma	1	F	38	Topical treatment	30	No	Surgery	Recovered
Bladder carcinoma	1	M	52	PUVA, retinoids	15	Yes	Surgery	Recovered
Colon carcinoma	1	M	45	Unknown	11	Yes	Surgery	Unknown
Thyroid carcinoma	1	F	34	PUVA	4	Yes	Surgery	Recovered
Lymph node metastasis of unknown origin	1	M	72	Unknown	3	No	Unknown	Unknown

*The causal relationship with CyA has not been established.

[†] Includes three patients not participating in Sandoz Pharma programme.

[‡] Patient not participating in Sandoz Pharma programme.

PUVA = psoralen photochemotherapy; MTX = methotrexate.

TABLE 4. Lymphoproliferative disorders* arising with CyA treatment in 842 patients participating in the Sandoz Pharma Ltd clinical study programme

Type	*n*	Gender	Age (years)	Previous therapy	Duration of treatment (months)	Discontinuation	Outcome
T-cell lymphoma	1	M	59	PUVA, etretinate	1·5	Yes	Spontaneous regression
B-cell lymphoma	1	F	55	Topical treatment	6	Yes	Spontaneous regression
Benign lymphocytic infiltration	3	M	36–58	Unknown, PUVA ± MTX, etretinate	0·5–4·5	2 yes, 1 no	Spontaneous regression

*The causal relationship has not been established.
PUVA = photochemotherapy; MTX = methotrexate.

of CyA treatment. The limits beyond which values were considered abnormal are given for each parameter.

Haematology. During CyA treatment, the percentage of patients with low haemoglobin concentrations increased slightly. These changes were independent of other haematological changes. A slight normochromic anaemia is a recognized side-effect of CyA.[13,14] The changes in the white blood cell count, seen only in a few patients, occurred mainly in those with intercurrent infections. A decrease in platelets was also seen only in a few patients and was clinically asymptomatic.

Biochemistry. The protocol of most of these studies required a reduction or discontinuation of the daily CyA dose if serum potassium, bilirubin and/or transaminases exceeded the upper normal limits.

Renal function. Only four parameters of renal function were analysed: serum magnesium, serum potassium, serum urea and serum uric acid. An increase in the latter may result from CyA-induced renal dysfunction which, in turn, may lead to hypomagnesaemia, hyperkalaemia and an increase in urea.

At baseline, only a few patients showed abnormalities of serum magnesium and potassium. During CyA treatment, the percentage of patients with low magnesium or high potassium concentrations increased to a maximum of 8·8% and 0·5%, respectively. The changes in serum potassium were related to neither initial dose nor duration of treatment. Doses were adjusted whenever serum potassium levels increased. In contrast, the frequency of hypomagnesaemia was clearly dose-dependent.

Increased levels of urea and uric acid were already present in 1·3% and 2·6%, respectively, of the patients at baseline. During CyA treatment, the incidence rose to 5·4% and 8·7%, respectively. However, gouty attacks were reported in only four out of 631 patients. These increases were dependent on the dose, but not duration, of treatment.

Hepatic function. The laboratory tests relating to hepatic function included serum albumin,

TABLE 5. Laboratory abnormalities in CyA-treated patients with psoriasis

Parameters	Abnormal limits	n	Duration of treatment (weeks)		
			Baseline	1–4	9–12
Haematology					
Haemoglobin: M	< 8·7 mmol/l	553	10·1	11·4	12·8
F	< 7·5 mmol/l				
WBC	< 3200/mm^3	566	0·2	0·6	0·3
Platelets	< 100,000/mm^3	548	0	0	0·3
Biochemistry					
Renal function					
Potassium	> 5·6 mmol/l	571	0·2	0·5	0
Magnesium	< 0·9 mmol/l	471	1·1	8·8	2·4
Urea	> 20% aunl	227	1·3	5·4	5·1
Uric acid: M	> 506 μmol/l	499	2·6	8·7	5·8
F	> 406 μmol/l				
Liver function					
Albumin	< 10% blnl	322	0	3·0	0
Alkaline phosphatase	> 25% aunl	570	3·3	4·6	8·1
Bilirubin	> 25% aunl	571	1·1	4·3	4·7
Gamma-GT	> 25% aunl	334	4·8	4·5	2·4
SGOT	> 25% aunl	570	2·5	2·7	5·7
SGPT	> 25% aunl	347	1·4	5·1	1·3
Metabolism					
Cholesterol	> 20% aunl	262	1·9	6·7	5·6
Triglycerides	> 20% aunl	262	9·9	23·6	16·9

aunl = above upper normal limit; blnl = below lower normal limit; n = number of patients examined; Gamma-GT = gamma-glutamyl transpeptidase; CyA dose = 1·25–7·5 mg/kg/day.

alkaline phosphatase, bilirubin and three transaminases. As already mentioned, an increase of the latter two required dose adjustments according to the study protocols. During treatment, 3% of patients showed low albumin concentrations, an effect which was not related to time. This decrease in albumin concentration may have contributed to the development of the oedema in some of the patients taking CyA. Approximately 8% of patients receiving CyA showed increases in alkaline phosphatase which was apparently related to time, but not to dose. Only in an exceptional case was an increase in alkaline phosphatase observed in combination with an increase in bilirubin and/or SGOT or SGPT, suggesting that the increase in alkaline phosphatase was not hepatic, but related to an osseous isoenzyme. This finding has been described for renal transplant recipients treated with CyA,[15] although the pathogenesis and clinical relevance of these alkaline phosphatase changes remain unknown.

A greater number of patients taking CyA had increases in serum bilirubin levels and these changes were dependent on the initial dose, but not necessarily on the duration of treatment.

In a few patients, at least one of the transaminases were already elevated at baseline and, during treatment, these values rose and required an adjustment of the daily dose of CyA. The number with abnormal liver function tests was related to the dose, but not necessarily to the

TABLE 6. Mean changes in serum cholesterol and triglycerides in CyA-treated patients with psoriasis

CyA doses* (mg/kg/day)	Cholesterol		Triglycerides	
	n	%	*n*	%
1·25	12	2·7	12	−9·7
2·5	111	7·9	122	13·2
5	22	9·8	22	21·7

12 weeks of treatment; pooled data; *n* = number of patients.
*Initial doses.

duration of treatment. No clear pattern was discernible involving the transaminases in association with elevation of the bilirubin. In some patients, the increase of one of the transaminases occurred in isolation and/or was transient. For several patients with concomitant increases in bilirubin and transaminases, these changes were not related to CyA, but due to ethanol intake. No patient was withdrawn from CyA treatment because of abnormalities of liver function. However, in one patient the drug was discontinued because of jaundice, but this was not drug-related.

Metabolic functions. The effect of CyA on blood lipids was investigated in a total of 262 patients. The parameters examined were serum cholesterol and triglycerides. These were determined in patients who were not on a special diet and were not always performed under fasting conditions. At baseline, approximately 2% of patients showed increased cholesterol levels and approximately 10% had elevated triglyceride levels.

During the first weeks of treatment, the number of patients who had abnormal cholesterol and triglyceride levels increased. However, the number showing an increase of cholesterol of more than 20% above the upper normal limits remained essentially unchanged during the 12-week treatment period. The number of patients with increased triglyceride levels doubled during the first 4 weeks of treatment, but gradually decreased thereafter. Further analysis produced the following results.

1. After 12 weeks of treatment, the average increases in cholesterol and triglycerides over baseline were dependent on the initial dose (Table 6). In patients receiving 5 mg/kg/day, the average increase in cholesterol and triglycerides over baseline was 10% and 22%, respectively. In patients treated for up to 48 weeks, the two lipids did not increase further. This indicates that, after an initial rise with CyA, cholesterol and triglyceride levels stabilize.
2. Assessing the CyA-treated patients individually, cholesterol blood levels varied over time. Except in one patient, no gradual and continuous increase in cholesterol level was observed, and the triglyceride levels showed a similar pattern.
3. No association was observed between the increases in cholesterol and triglycerides. Patients who had high cholesterol and triglyceride levels did not increase further during CyA treatment. There was no association between the extent of the increases in the two lipids and rises in diastolic blood pressure. However, the latter analysis did not consider a possible bias due to antihypertensive therapy.
4. In the few patients who were examined 12 weeks after the end of treatment, the average cholesterol and triglyceride levels approached baseline values.

DISCUSSION AND CONCLUSIONS

The adverse events observed in patients treated with CyA for severe psoriasis are very similar to those of transplant patients treated with CyA.[16] Paraesthesia and tremor as well as hypertrichosis, gingival hyperplasia and gastrointestinal disorders were the most common side-effects. These were usually transient and in only a few cases was the drug discontinued. Serious adverse events such as epileptic seizures and haemolytic–uraemic syndrome, seen occasionally in transplant patients treated with CyA, were not reported in the psoriatic patients. Infections were not a particular problem.

However, there are problems regarding safety with the CyA treatment of psoriasis, and these are renal dysfunction, hypertension and a possible increase of malignancies, in particular, squamous cell carcinomas of the skin and lymphoproliferative disorders. According to experimental data, CyA is neither mutagenic nor carcinogenic.[17,18] However, it is suspected that the incidence of tumours in CyA-treated psoriatic patients is higher than in the normal population. This may be associated with any drug possessing immunosuppressive action. The squamous cell carcinomas occurred almost exclusively in those patients previously treated with PUVA and/or methotrexate. These tumours can be detected early by physical examination, and the chances of a cure are good following cessation of CyA and institution of conventional antitumour therapy. The lymphoproliferative disorders regressed spontaneously on stopping CyA.

Apart from serum creatinine, an important indicator of renal dysfunction in association with CyA treatment,[5] the observed changes in laboratory parameters were generally not relevant except for changes in electrolytes and uric acid. This, however, is not unexpected as they may result from renal dysfunction. The changes in liver function tests were reversible and there were no clinical symptoms of liver toxicity. The changes in cholesterol and triglycerides showed wide fluctuations over time in individual patients and were, in all cases, reversible. As lipid determinations were not carried out under strictly controlled conditions, the clinical relevance of these findings remains unclear. Extensive laboratory monitoring is required during CyA treatment of patients with psoriasis. If relevant abnormalities are detected, treatment should be adjusted accordingly.

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Renal function and blood pressure in psoriatic patients treated with cyclosporin A

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SUMMARY

Serum creatinine and blood pressure were measured in patients who had severe psoriasis and who were treated with cyclosporin A (CyA) in initial doses of 1·25 mg ($n = 34$), 2·5 or 3 mg ($n = 314$), or 5 ($n = 215$) mg/kg/day. Of the 563 patients involved, 201 were treated for more than 3 months, and 100 received CyA continuously for 12 months or more. Sixty-eight additional patients were included as controls and received placebo ($n = 42$) or etretinate ($n = 26$). At doses of 2·5 and 5 mg/kg/day, CyA induced slight but significant dose-dependent increases in serum creatinine and blood pressure. Creatinine increases of 50% or more over baseline values were detected in 4% of the patients receiving 2·5 mg/kg/day and in 13% of those receiving 5 mg/kg/day. After an initial rise during the first weeks of treatment, mean creatinine level remained stable over 1 year provided that the CyA dose was reduced whenever creatinine levels increased by 30% or more over baseline. The incidence of hypertension was 10·6% and did not vary whether the CyA dose was 2·5 or 5 mg/kg/day. The first elevated blood pressures were recorded early after starting CyA therapy (median: 1 month). However, 3 months after stopping treatment, the increases in creatinine as well as in blood pressure were reversible and the levels did not significantly differ from baseline values.

Cyclosporin A (CyA) has proved to be effective in inducing remission of severe psoriasis.[1,2] Although low-dose CyA is effective, the quickest results are obtained with a dose of 5 mg/kg/day.[3-5] However, the use of CyA is limited by the occurrence of renal dysfunction. A decrease in glomerular filtration rate and renal plasma flow, resulting in an increase of serum creatinine and urea, is the most frequent adverse event. Renal dysfunction is dose-dependent, but little is known at present of the incidence and intensity of this side-effect when using moderate or low doses of CyA over long periods of time. The same applies to hypertension. It has been shown that the risk of developing irreversible structural alteration in the kidney or of irreversible renal dysfunction is closely dependent not only on the dose, but also on the intensity of CyA-induced renal dysfunction.[6-8] Therefore, information on CyA-associated

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renal dysfunction and hypertension is essential for determining the risk:benefit ratio of the drug in psoriasis and for selecting the best therapeutic strategy.

This paper presents a pooled analysis of the changes in serum creatinine and blood pressure for 1 year or more in a large series of CyA-treated psoriatic patients in therapeutic trials conducted by Sandoz.

PATIENTS AND METHODS

Six-hundred and thirty-one adult patients were included (mean age: 43 years; range: 18–70). All were participants in 10 studies involving more than 50 dermatology departments in Europe, and all had severe psoriasis as defined by a Psoriasis area and Severity Index (PASI)[9] score $\geqslant 18$. The mean PASI score at inclusion was 25 and the duration of psoriasis was 14 years.

Treatment

Controls. Of the 631 patients, 68 were included in control groups of four double-blind studies. In three of these studies, the controls received placebo ($n = 42$) and, in one study, etretinate ($n = 26$). The duration of placebo treatment was 1 month whereas, for etretinate, it was 3 months. The etretinate dose was 0·75 mg/kg/day for 2 weeks, followed by 0·50 mg/kg/day for 6 weeks; patients were then 'weaned' over a further 4 weeks.

Cyclosporin A dose. Cyclosporin A was administered to 563 patients at a maximum dose of 5 mg/kg/day. Apart from 10 patients who participated in the early pilot studies, patients were randomly allocated to a dose group in either placebo-controlled or open dose-finding studies. The initial CyA dose (divided into two daily doses) was 1·25 mg/kg/day in 34 patients, 2·5 or 3 mg/kg/day in 314 patients, and 5 mg/kg/day in 215 patients. The CyA dose was further adapted according to efficacy and renal dysfunction. In cases of inefficacy, the dose was increased, but without exceeding 5 mg/kg/day, after 1–3 months of treatment. Thus, for each patient, only the period before the dose increase was considered for analysis of creatinine changes in relation to dose. In cases of renal dysfunction, defined as a creatinine increase of more than 30% over patient's own baseline, a CyA dose reduction of 25–50% was required by study protocols.

Duration. All trials consisted of induction and post-treatment observation periods of 3 months each. Treatment was, however, shorter in cases of persistent inefficacy or serious adverse events. Two studies were designed to include long-term maintenance CyA treatment (12–16 months) in patients showing clinical success. These two long-term studies involved 361 patients of the 563 receiving CyA. Cyclosporin A treatment was for 4 or 5 months in 201 patients, 6 months in 183, 9 months in 159 and 12 months or more in 100 patients. Eighty-three patients who started with CyA at 5 mg/kg/day entered the maintenance period at that dosage before beginning a 3-month tapering of the dose to reach 2·5 mg/kg/day; 77 patients receiving 2·5 mg/kg/day continued at that dosage. Forty-one patients, who had initially failed to respond during the induction period with CyA at 2·5 mg/kg/day, had clinical success on increasing the dose to 5 mg/kg/day. During the maintenance phase, these patients followed the same treatment-tapering strategy as those taking 5 mg/kg/day. If a patient relapsed, the dose was again increased to 5 mg/kg/day for the remainder of the maintenance period. Because of these many dose adjustments, no clear dose-effect relationship with renal function and blood pressure could be analysed during the maintenance period. The results were reported for the whole group with the mean CyA dose administered.

Post-treatment observation. In 100 patients, serum creatinine was measured for 3 months after stopping the CyA treatment, which had been preceded by a 1–3-month tapering period. Of these 100 patients, 45 had received CyA for 3 months or less, 24 for 4–6 months, 15 for 7–12 months, and 16 for 12 months or more. The blood pressure was measured in 75 patients.

Creatinine measurements

Evaluation of CyA-associated renal dysfunction was based on serum creatinine levels, and the results expressed in two ways. In one, the concentration of serum creatinine was reported. This classical evaluation lacks precision as an estimate of true renal function [glomerular filtration rate (GFR)] as each patient's weight (muscular mass), height, gender and age must also be considered. Several formulas for GFR estimation from serum creatinine have been reported, but none has proved to be completely reliable.[10,11] Another method was to evaluate the changes in serum creatinine concentration by comparing creatinine levels before and during CyA treatment. The results were expressed as the percentage increase of serum creatinine over patient's own baseline values. In addition to standardizing the changes of serum creatinine for each patient, this calculation also avoided the interlaboratory variations of creatinine measurement.

In the first 3-month induction period, serum creatinine was measured every week for the first month and then at the end of Weeks 6, 8 and 12. Instead of a value taken at a fixed time-point, the maximum serum creatinine concentration (or increase) with CyA treatment was selected as a key parameter. This was because renal dysfunction was often transient as the dosage was reduced each time it occurred. The maximum value during the treatment period for each patient was thus a true reflection of renal dysfunction.

As already mentioned, not all patients received 3 months of CyA treatment as some discontinued the treatment because of clinical inefficacy. Thus, the last creatinine value measured during the induction period was selected for analysis instead of the creatinine level at 3 months. During the maintenance and 3-month post-treatment periods, serum creatinine was measured every 4–6 weeks.

Blood pressure

During the induction period, modifications in systolic or diastolic blood pressure were expressed as changes in mm Hg in comparison with baseline measurements. The incidence of abnormal blood pressure was recorded in patients who participated in the two long-term (induction plus maintenance) studies which lasted for more than 3 months. This was because one of the criteria for defining hypertension was the persistence of elevated blood pressure for at least 4 consecutive months (see below).

Elevated blood pressure was defined according to the World Health Organization (WHO) definition (systolic BP $\geqslant$ 160 mm Hg or diastolic BP $\geqslant$ 95 mm Hg).[12] However, in the studies reported here, blood pressure was measured only once, after a few minutes rest, at each clinical visit whereas the WHO definition requires the mean of three measurements to determine true blood pressure levels. If a patient showed persistently elevated blood pressures (at two consecutive visits with an interval of 1 month between each visit), the protocols recommended

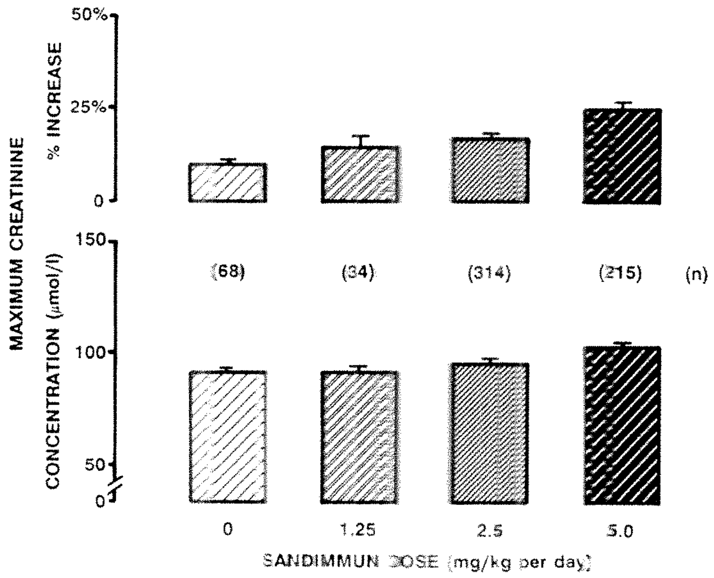

FIGURE 1. Maximum creatinine in the first 3 months of treatment according to Sandimmun® (CyA) dose, serum creatinine concentration (lower) and percentage increase over baseline (upper). The 0 mg/kg/day group included placebo or etretinate (n = 26). Means ± SEM. ( ) = number of patients.

starting antihypertensive therapy. This was much stricter than the WHO recommendations for antihypertensive therapy, which are based on diastolic blood pressure (mean mm Hg of three measurements: > 105 at one visit; or 100–105 at two visits with an interval of 1 month; or 95–99 at four visits with an interval of 4 months).[12] Only these latter patients were considered in this paper to have hypertension. Not all of the patients taking CyA who received antihypertensive therapy for the first time fulfilled the WHO criteria for starting such medication. Therefore, results were analysed separately according to each of the two criteria.

Statistical tests

These results are reported as means ± SEM. The statistical significance of the difference between the groups was assessed using Student's t-test, and the frequency of abnormalities was compared between groups using Fisher's exact test.

RESULTS

Creatinine

Induction period (initial 3 months). Cyclosporin A induced a slight, but significant, dose-dependent increase of the serum creatinine (Fig. 1). In the controls, the maximum creatinine increase above baseline was 10 ± 1%. This change can be explained by the slight changes in the patient's metabolic status from one visit to another and by technical variations in the measurement of creatinine. Assessment of the maximum creatinine levels over a specific period of time will reflect the maximum technical variations in such measurement.

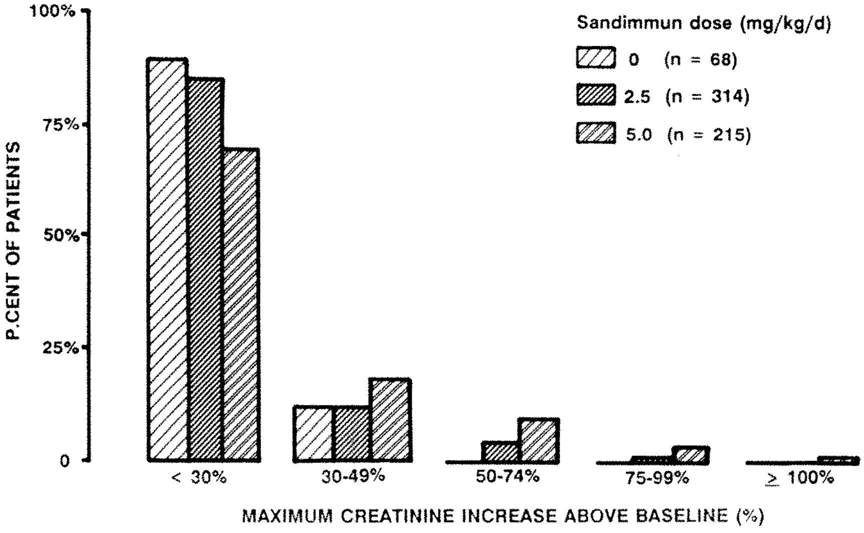

FIGURE 2. Incidence of renal dysfunction during the first 3 months of Sandimmun® (CyA) treatment according to dose. The 0 mg/kg/day group included placebo or etretinate ($n = 26$).

Creatinine levels increased by 15 ± 3% (not significant *vs* controls) with CyA 1·25 mg/kg/day, by 17 ± 1% ($P < 0{\cdot}01$ *vs* controls) with 2·5 mg/kg/day, and by 25 ± 1% ($P < 0{\cdot}001$ *vs* controls and *vs* the 2·5 mg group) with 5 mg/kg/day. Corresponding concentrations of serum creatinine were 92, 96 and 102 μmol/l on average respectively.

In the control group, nine patients (13%) had serum creatinine levels which exceeded the uppermost limit of normal (for the corresponding laboratory), but none exceeded 130 μmol/l. No patient exceeded the normal value with CyA 1·25 mg/kg/day, but 30 patients (10%) taking 2·5 mg/kg/day and 52 patients (24%) taking 5 mg/kg/day showed levels above normal. Serum creatinine concentrations greater than or equal to 130 μmol/l were detected in 3% of the patients taking CyA 2·5 mg/kg/day and in 9% of those receiving 5 mg/kg/day. During this period, no creatinine level above 200 μmol/l was detected.

The incidence of renal dysfunction was also reported according to the percentage increase of serum creatinine above baseline values (Fig. 2). In the control group, no patient had an increase equal to or greater than 50% whereas such an increase was found with 13 patients (4%) receiving CyA 2·5 mg/kg/day and 28 patients (13%) taking 5 mg/kg/day. Because of the various dose adaptations, changes in serum creatinine were lower than maximum at the last visit: 0 ± 1% in controls; 5 ± 4% in the CyA 1·25 mg/kg/day group; 5 ± 1% in the 2·5 mg/kg/day group; and 11 ± 1% in the 5 mg/kg/day group.

Maintenance period (4–16 months). Mean creatinine increases were 12 ± 1% after 6 months of treatment ($n = 183$) and 9 ± 2% after 9 months of treatment ($n = 159$). The corresponding mean CyA doses were 3·3 mg and 3·2 mg/kg/day, respectively. One hundred patients were treated continuously for 1 year or more. The time course of mean serum creatinine for these patients, expressed as percentage increases over baseline, is summarized in Fig. 3. After a slight but significant increase in serum creatinine during the first weeks of treatment, no significant change was subsequently detected. However, the mean CyA dose was lower during these 4–12

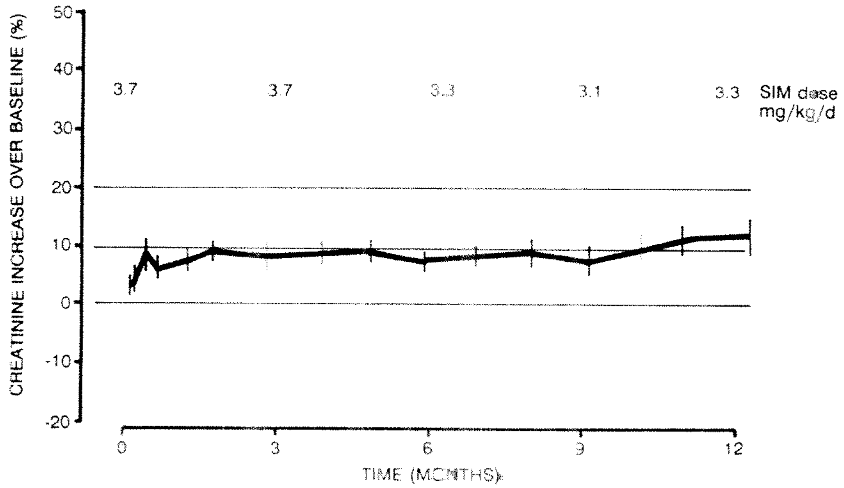

FIGURE 3. Time course of serum creatinine in the subgroup of 100 patients continuously treated with SIM (CyA) for 12 months or more. Means ± SEM. The corresponding mean SIM dose/month is shown.

months than in the first 3 months. Although not significant, the trend towards a higher mean creatinine level at 12 compared with 9 months may be due to an increase in CyA dose in some patients because of a recurrence of psoriasis.

Post-treatment period. In a subset of 100 patients, serum creatinine was measured in the 3 months after stopping CyA treatment (Fig. 4). At 1, 2 and 3 months after stopping, the creatinine level was 4 ± 2%, 3 ± 2% and 2 ± 2% higher (not significant) than baseline, respectively. This course varied slightly according to the dose of CyA. In patients receiving 2·5 mg/kg/day, the maximum and last creatinine measurements taken during CyA treatment were lower than for those receiving 5 mg/kg/day. Consequently, the reversibility of the creatinine increase was quicker with CyA 2·5 mg/kg/day than with 5 mg/kg/day.

Three months after stopping CyA, the changes in serum creatinine in comparison to baseline were similar to those observed at the last clinical visit of the non-CyA-treated controls (Fig. 5). Only four patients (4%) retained creatinine levels 30% higher than at baseline and, in two of these, a wide variation in creatinine levels was observed at the three post-treatment visits (ranging from −5% to 52%) which was probably due to variability in measurement techniques. The remaining two patients showed consistent increases in creatinine levels by 31% (97 μmol/l) and 47% (88 μmol/l), respectively. Both patients had received CyA at 5 mg/kg/day. However, it is not always easy to differentiate persistent renal dysfunction from slow normalization, which has sometimes been observed, or from an inaccurate baseline measurement.

Blood pressure

Mean values. After 1 month of treatment, slight increases in diastolic blood pressure were

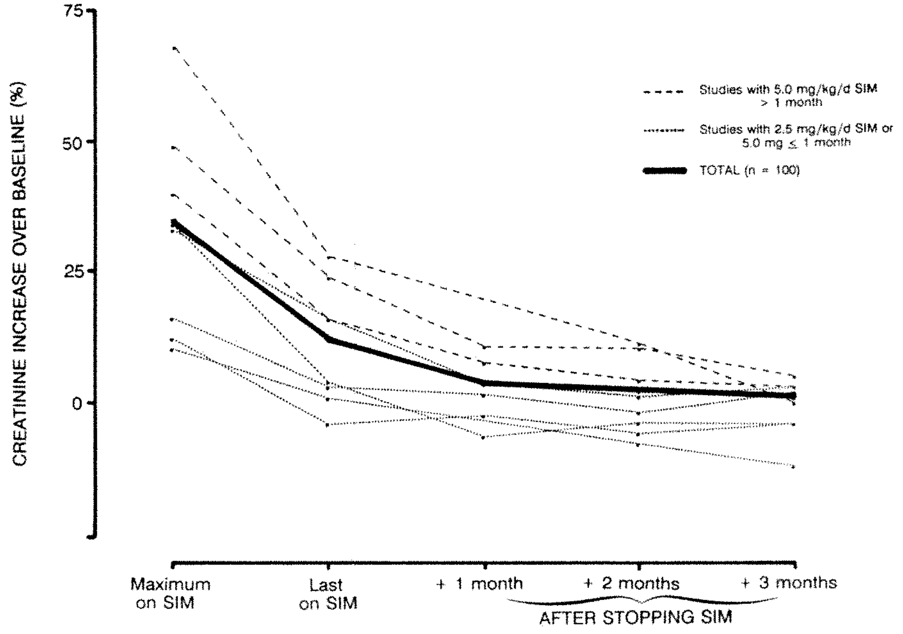

FIGURE 4. Time course of serum creatinine in the 3 months after stopping SIM (CyA) treatment.

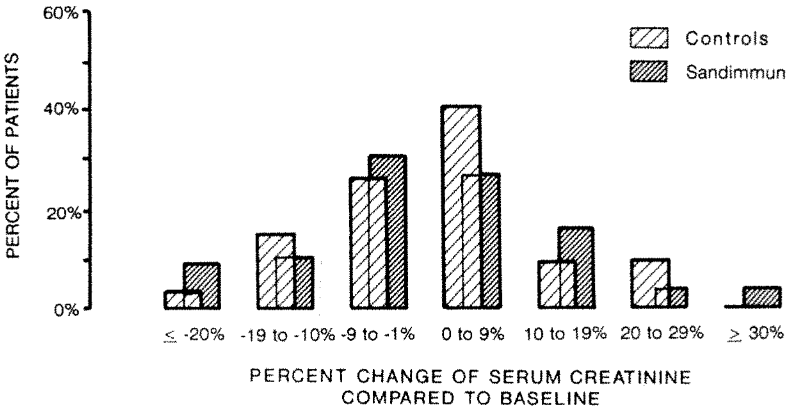

FIGURE 5. Distribution of patients according to serum creatinine measured 3 months after stopping Sandimmun® (CyA) ($n = 100$) in comparison to values taken at the last visit in controls ($n = 68$). The controls included patients who received placebo as well as etretinate ($n = 26$).

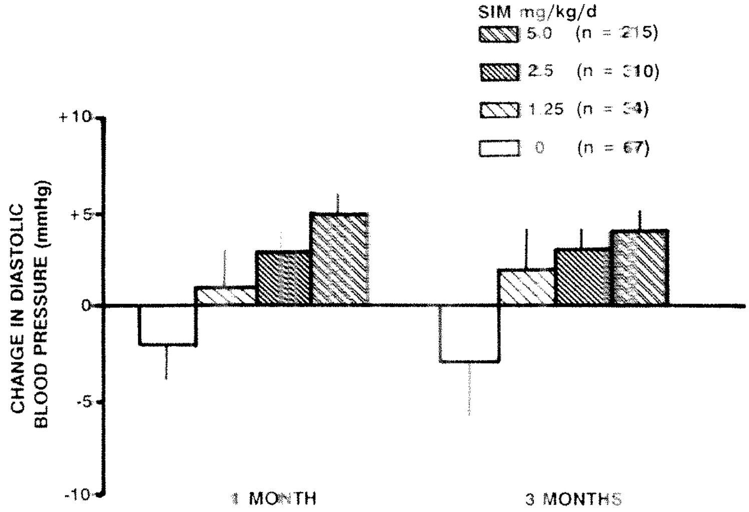

FIGURE 6. Changes in diastolic blood pressure (mm Hg) in comparison to baseline after 1 and 3 months of SIM (CyA) treatment according to dose. The 0 mg/kg/day group included patients who received placebo as well as etretinate ($n = 26$). Means ± SEM.

observed: 1 ± 2 mm Hg with CyA 1·25 mg/kg/day; 2·8 ± 0·6 mm Hg with 2·5 mg/kg/day; 5·2 ± 0·8 mm Hg with 5 mg/kg/day ($P < 0{\cdot}02$ *vs* 2·5 mg/kg/day) compared with a decrease of 1·8 ± 1·5 mm Hg in controls ($P < 0{\cdot}01$ *vs* CyA 2·5 or 5 mg/kg/day groups) (Fig. 6). No progression of the increase in diastolic blood pressure was seen between 1 and 3 months, and changes in systolic blood pressure were similar.

Hypertension. Of the 361 patients included in the long-term studies, 32 had elevated blood pressure at baseline and a further seven were already receiving antihypertensive medications before CyA starting treatment.

Of the 322 patients who initially did not have elevated blood pressure, 34 (10·6%) developed hypertension, nine of whom did not receive antihypertensive medication. This effect was independent of the initial dosage of CyA as the percentage of patients involved was 10% of those taking 2·5 mg/kg/day and 11·9% of those taking 5 mg/kg/day (not significant). In this subgroup of 34 patients, no differences were seen in blood pressure increases in relation to the dosages. Maximum mean systolic/diastolic blood pressure was 164/109 mm Hg with CyA 2·5 mg/kg/day and 170/107 mm Hg with 5 mg/kg/day (not significant). In these patients, the first elevated blood pressure measurement was taken very shortly after starting CyA treatment (median: 1 month). Only four of these 34 patients showed no blood pressure rises during the first 3 months of treatment.

Of the 322 patients without elevated blood pressures initially, a total of 38 (11·8%) started antihypertensive therapy. Among these patients, antihypertensive medications were inappropriately prescribed for 13 who fulfilled neither the protocols nor the WHO criteria for antihypertensive therapy. No difference was observed according to the initial CyA dosages as the percentage of patients receiving antihypertensive medications was 11·8% of the 221

TABLE I. Blood pressure before and 3 months after discontinuation of CyA treatment (means ± SEM)

	Before CyA		After stopping CyA	
	Systolic (mm Hg)	Diastolic (mm Hg)	Systolic (mm Hg)	Diastolic (mm Hg)
Total (*n* = 75)	130 ± 2	82 ± 1	129 ± 2	82 ± 1
Elevated BP at baseline				
Yes (*n* = 8)	146 ± 4	95 ± 1	138 ± 5	86 ± 3
No (*n* = 63)	127 ± 1	80 ± 1	127 ± 2	81 ± 1
Elevated BP with CyA				
Yes (*n* = 28)	134 ± 2	86 ± 1	137 ± 2	86 ± 2
No (*n* = 47)	127 ± 2	80 ± 1	124 ± 2	79 ± 1
Antihypertensive therapy and CyA				
Yes (*n* = 16)	132 ± 2	87 ± 2	139 ± 3	88 ± 3
No (*n* = 59)	129 ± 2	81 ± 1	126 ± 2	80 ± 1

BP = blood pressure.

patients who initially received CyA 2·5 mg/kg/day and 11·9% in the 101 patients taking 5 mg/kg/day.

Of the 32 patients who had elevated baseline blood pressure, 19 (59·4%) had hypertension with CyA. However, of the seven patients already receiving antihypertensive medication before treatment (and therefore showing no rises in blood pressure at baseline), only one lost the control of hypertension on receiving CyA.

Effect of antihypertensive therapy. Receiving antihypertensive medication (mainly calcium antagonists) while taking CyA resulted in a decrease in mean systolic/diastolic blood pressure from 156/102 mm Hg to 149/92 mm Hg, although the mean blood pressure was higher while taking antihypertensive medication concomitantly with CyA than at baseline (139/85 mm Hg). Blood pressure was well controlled by the antihypertensive therapy (systolic BP < 160 mm Hg and diastolic BP < 95 mm Hg) in 24 out of 38 patients (63%).

Post-treatment visits. For the 75 patients whose blood pressures were measured after stopping CyA therapy, mean systolic/diastolic blood pressure was 129/82 mm Hg during the post-treatment period compared with 130/82 mm Hg before CyA treatment. Comparative analysis of the mean blood pressure values in the patients with or without elevated blood pressure at baseline, in those with elevated blood pressure on receiving CyA and in those who received antihypertensive therapy (Table I) revealed no differences between baseline and post-treatment values. Nine (12%) of these 75 patients had raised blood pressures before CyA therapy which was reduced to six (8%) patients after treatment was discontinued.

Relationship between blood pressure and renal dysfunction. Renal dysfunction, defined as a maximum creatinine increase of 50% or more over baseline, was seen in eight (24%) out of 34 patients with hypertension compared with 15 (5%) out of the 288 patients who had not developed hypertension on receiving CyA ($P = 0{\cdot}001$). If renal dysfunction was defined as a

TABLE 2. Maximum creatinine increase and hypertension according to previous therapies with or without retinoids or methotrexate (means ± SEM)

	Creatinine increase (% over baseline)		Incidence of hypertension*	
	Pre-treatment	No pre-treatment	Pre-treatment	No pre-treatment
Retinoids				
2·5 mg/kg/day	(n = 132) 16 ± 1	(n = 110) 19 ± 2	12/107 (11%)	10/87 (11%)
5 mg/kg/day	(n = 63) 31 ± 3	(n = 54) 24 ± 3	8/51 (16%)	4/39 (10%)
Total	(n = 195) 21 ± 1	(n = 164) 21 ± 1	20/158 (13%)	14/126 (11%)
Methotrexate				
2·5 mg/kg/day	(n = 60) 16 ± 2	(n = 182) 18 ± 1	5/38 (13%)	17/157 (11%)
5 mg/kg/day	(n = 33) 28 ± 4	(n = 84) 28 ± 3	5/27 (19%)	7/62 (11%)
Total	(n = 93) 20 ± 2	(n = 266) 21 ± 1	10/65 (15%)	24/219 (11%)

*Results of patients without elevated blood pressure at baseline.

creatinine increase of 30% or more over baseline, incidences were then 44% and 18% (P = 0·001), respectively.

Influence of previous therapies

Information concerning previous therapies for psoriasis was available for 359 patients. During the induction period, no consistent effect of previous therapy with either methotrexate (n = 93) or retinoids (n = 195) was seen in the extent of creatinine increase over baseline nor in the incidence of hypertension (Table 2).

DISCUSSION

No detectable change in serum creatinine was observed with CyA 1·25 mg/kg/day in comparison to placebo whereas the 2·5 and 5 mg/kg/day doses resulted in a slight but significant dose-dependent increase. However, these changes were limited as the mean maximum creatinine concentration was 96 μmol/l in the CyA 2·5 mg/kg/day group and 102 μmol/l in the 5 mg/kg/day group during the first 3 months of treatment, values which are within the normal range. Considering the patients individually, 3% had creatinine levels greater than or equal to 130 μmol/l with 2·5 mg/kg/day, and 9% with 5 mg/kg/day. Creatinine increases of more than 50% over baseline were detected in 4% of patients receiving 2·5 mg/kg/day and in 13% of those taking 5 mg/kg/day.

The results of these studies are much less worrying than those involving higher doses of CyA as was used in the past for the treatment of psoriasis. With CyA at a dose of 14 mg/kg/day, the mean creatinine concentration was 120 μmol/l, a mean increase of 30% over baseline after only 1 month of treatment.[1] In one patient treated with 8 mg/kg/day and without adequate adaptation of dose, a rise in serum creatinine from 84 to 334 μmol/l was observed after 1 year of treatment.[13] The relevance of using creatinine changes as a measure of altered glomerular filtration rate (GFR) is controversial because of tubular excretion of creatinine in some pathological conditions.[14,15] However, this was only reported in patients with substantial reductions of GFR (less than 50 ml/min/1·73 m^2 on average) and increases in creatinine (more than 160 μmol/l on average). A serious fall in GFR despite unchanged and still normal serum creatinine levels, reported in two psoriatic patients treated with CyA 4·7 mg/kg/day, awaits confirmation.[16] This emphasizes the need for assessing intraindividual variability of GFR measurements in non-CyA-treated patients as with serum creatinine (Fig. 5). In addition, when GFR changes abruptly, it takes several days for the serum creatinine to reach a new steady state,[17] which may also explain some of the discrepancies between these two parameters.

Slight changes in serum creatinine levels detected with low-dose CyA are not clinically relevant in themselves, but are important as the extent of creatinine is considered to be a potential risk factor for CyA-associated structural alteration of the kidney. Cyclosporin A nephropathy was not detected in patients treated with 5 mg/kg/day or less[18,19] but, in patients receiving more than 7 mg/kg/day, the CyA dose and the extent of creatinine increase were found to be additive risk factors,[20] especially when creatinine was increased by more than 50% above baseline.[6,7] On its own, the 50% creatinine increase with CyA was also found to be a risk factor for persistent renal dysfunction after stopping treatment in patients with autoimmune diseases who were receiving a mean CyA dose of 8·8 mg/kg/day.[8]

Adaptation of CyA dosages, based on the serum creatinine, resulted in a stable creatinine level in 100 patients treated continuously for 1 year or more with a mean dose of CyA at 3·3–3·7 mg/kg/day. None of these patients developed protracted renal dysfunction, at variance with some cases observed after initial high doses of CyA but without dose adaptation according to serum creatinine levels.[13,21,22]

No significant difference in mean creatinine concentration in comparison to baseline was observed 3 months after stopping treatment. The return to baseline level was quicker in those treated with CyA 2·5 mg than in those taking 5 mg/kg/day. Three months after stopping CyA, only two patients had not returned to baseline levels. However, it was not possible to determine whether this was a slow return to 'normal', an inaccurate baseline creatinine value or a slight residual renal dysfunction. Interpreting slight changes in serum creatinine is not easy because of the wide range of normal variations in creatinine concentrations, as seen among the psoriatic controls (Fig. 5). Coefficients of variation in creatinine measurements have been reported between 3% and 18·5% using colorimetric as well as enzymatic techniques.[11]

The validity of assessments of the reversibility of CyA-associated renal dysfunction has been questioned because of the possibility of compensating glomerular hyperfiltration and/or hypertrophy.[23] However, this possibility is very unlikely in the present study for two reasons. Glomerular hypertrophy is the hallmark of a severely reduced number of glomeruli, and glomerular fibrosis is not a feature of CyA-induced nephropathy[24] except in the rare event of severe alterations as observed among cardiac transplant recipients.[23] No CyA nephropathy was seen in the first 14 kidney biopsies performed in patients with psoriasis treated with CyA[19] nor in a series of 21 patients similarly treated with low-dose CyA ($\leqslant 5$ mg/kg/day) for various autoimmune diseases.[18] A slight (3–5 mm Hg) dose-dependent increase in systolic and diastolic blood pressures was observed. However, the incidence of hypertension was not dose-related and was approximately 10–15% in both groups. These results suggest that a proportion of the population is particularly sensitive to CyA and is prone to develop hypertension even with low-dose CyA. Thus, hypertension should first be treated with antihypertensive therapy rather than with a reduction in the dose of CyA. Two-thirds of the patients receiving antihypertensive medication (mainly calcium antagonists) showed satisfactory blood pressure control. Taken together, this is a strong argument against starting CyA treatment in patients with uncontrolled hypertension. The incidence of hypertension in CyA-treated psoriatic patients is in line with that described for other autoimmune indications.[8]

An association was found between hypertension and renal dysfunction, although half of the patients with hypertension had no relevant renal dysfunction. This has also been observed with CyA treatment in other conditions.[25] Increased blood pressure was reversible within 3 months of discontinuation of CyA. Previous therapy with methotrexate or retinoids did not modify the effect of CyA on blood pressure or renal function.

Finally, using low-dose CyA in the treatment of psoriasis[26] should avoid clinically relevant renal impairment even with long-term therapy, provided that the creatinine levels and blood pressure are monitored at regular intervals.

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Renal side-effects of cyclosporin A

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SUMMARY

Cyclosporin A (CyA) has multiple effects on the kidney. The drug can cause changes in either the tubular or vascular system which may be either functional or structural, reversible or irreversible and frequent or seldom. Functional changes to the tubules and vessels are common, occur at low doses and are reversible, while structural changes to the tubules and vessels are rare, occur at high doses and are not always reversible. The changes of importance are vascular. By harnessing two strategies to restrain the development of side-effects—limiting the dose and restricting the functional changes—it is possible to avoid irreversible damage to the kidney in patients treated with CyA.

To understand the different types of actions that CyA can exert on the kidney and to place these side-effects into a clinical perspective, a system of classification is needed. This framework cannot be adapted from those already in use to describe other nephrotoxic agents, as the effects of CyA on the kidney differ from those normally encountered with the other substances usually referred to as 'nephrotoxic'.

The term 'nephrotoxicity' is not appropriate for describing the actions of CyA on the kidney. Although the term indicates that the kidney is the organ most susceptible to adverse effects, it does not specify the types of alterations that occur or their relevance. As the term is widely used to describe the action of a drug with adverse effects on the kidney, there is a danger that the side-effects caused by CyA may be confused with those seen with the classical nephrotoxic agents.

The cause of the different renal side-effects and the relationships between them have not yet been established. Consequently, any system of classification must of necessity be descriptive. This has the advantage of preserving neutrality and avoids prejudicing any future discoveries concerning the mechanisms of the different side-effects and the sequence of their development.

TYPES OF CHANGE

The kidney is a complex organ composed of two major tissue types. The vessels, consisting mostly of smooth muscle cells, are contractile and regulate blood pressure and flow within the renal vasculature. These determine the haemodynamic functions of the kidney, such as renal perfusion and glomerular filtration. The tubules, consisting of epithelial cells, transport small

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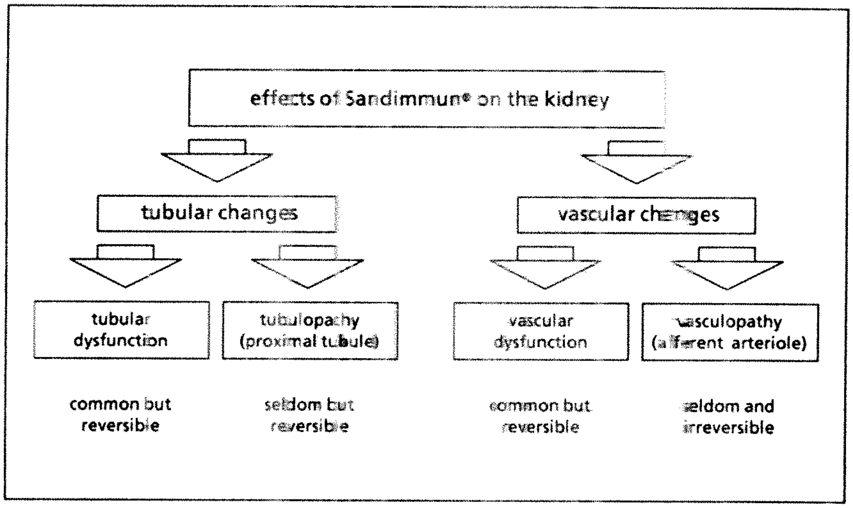

FIGURE 1. Tubular and vascular effects of CyA on the kidney.

molecules either from or into the urine. By this process of reabsorption or secretion, the tubules regulate the composition of body fluids.

Both of these tissue types, the vessels and tubules, can be affected by the two types of alterations during CyA treatment. There may be changes in function that are not accompanied by any visible change in structure, or changes in the structure that are usually associated with changes in function. Hence, there are four possible types of alteration to the kidney that can occur with CyA (Fig. 1).

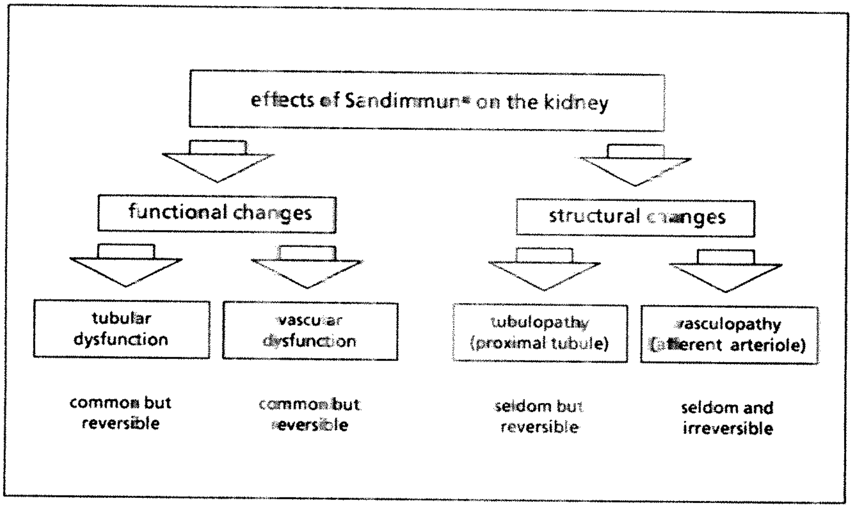

FIGURE 2. Functional and structural effects of CyA on the kidney.

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Participants

The following participants attended the cyclosporin A (Sandimmun®) consensus meeting 26-28 February 1990 and actively participated in the formulation of the consensus statement. The list is printed with the kind permission of the British Journal of Dermatology for distribution with copies of the Supplement.

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PREVALENCE

All four types of changes to the kidney have been seen during therapy with CyA but, as they are not always present at the same time, they have different prevalences. While changes in tubular function are common, changes in tubular structure are less so. Correspondingly, the changes in vascular function are common, but the changes in vascular structure are more rare. Thus, the changes in the kidney caused by CyA occur with two different prevalences (Fig. 1).

If the four types of kidney alteration are categorized according to the nature of the change rather than the type of tissue involved, the most common changes are seen to be functional. These occur frequently with CyA treatment, but are reversible on discontinuation of the drug. In contrast, changes in structure are seldom seen with CyA and are sometimes, but not always, reversible when treatment is discontinued (Fig. 2).

DOSE-DEPENDENCY

As can be inferred from the different prevalences of the kidney changes, the adverse effects of CyA show variable degrees of dose-dependency. Functional changes to both tubular and vascular systems are common and can be found at low dosages, whereas structural changes are more rare and occur only at higher dosages. In the past, when patients often received higher doses of CyA, structural changes were more frequent. Now that much lower doses are used, the incidence of these alterations has been greatly reduced.

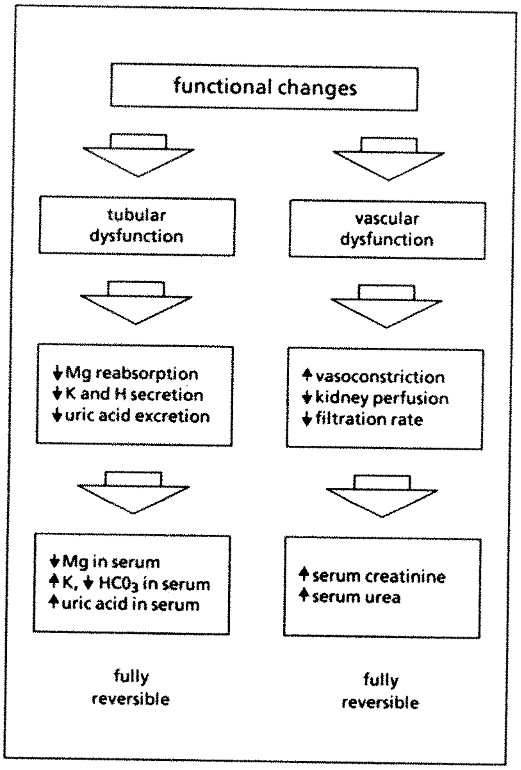

FIGURE 3. Details of functional changes with CyA.

The different dose-dependencies of these changes have two consequences. First, the structural changes in the vessels and tubules are always accompanied by the corresponding functional alterations; second, the functional changes often arise without the corresponding structural alterations. Thus, structural changes always follow functional alterations and are most often associated with them.

DESCRIPTION

The predominating changes in the tubules with CyA are changes in function (tubular dysfunction). These consist of a decrease in magnesium reabsorption, secretion of potassium and acid, and excretion of uric acid. This results in a decrease in magnesium and bicarbonate levels in serum, and an increase in potassium and uric acid levels. There is no decrease in sodium reabsorption and no loss of urinary concentrating power, as is characteristic of heavy-metal toxicity (such as with mercury, platinum, cadmium or uranium) or aminoglycoside toxicity (such as with gentamicin) (Fig. 3).

The changes that predominate in the vessels during CyA treatment are functional (vascular dysfunction). These result in an increase in vasoconstriction of the renal vessels. This leads to a decrease in renal perfusion, as seen by a decrease in renal blood or plasma flow, and also to a lowering of the glomerular filtration rate, resulting in an increase in serum creatinine and urea (Fig. 3).

Changes in tubular structure (tubulopathy) are less frequent with CyA. These are seen in

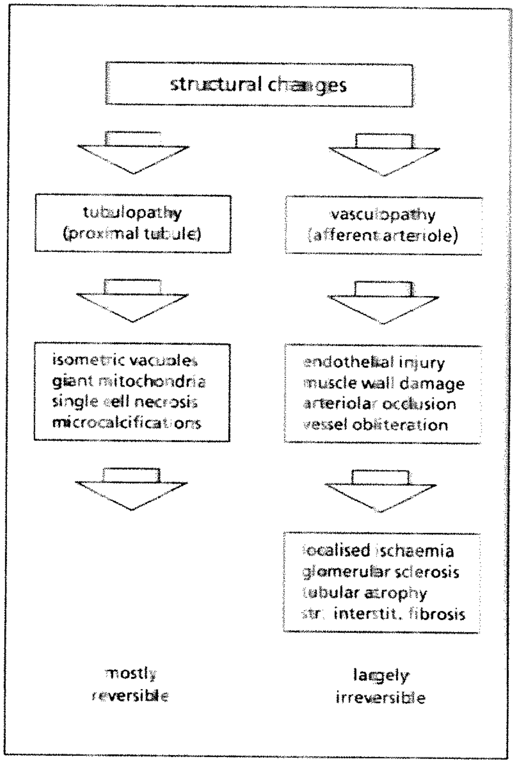

FIGURE 4. Details of structural changes with CyA.

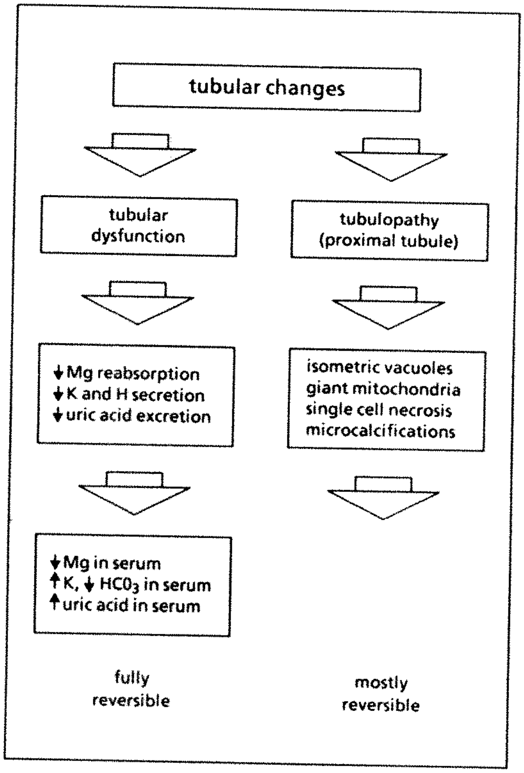

FIGURE 5. Tubular changes with CyA.

the proximal tubule as isometric vacuoles, the occasional giant mitochondrion or isolated single cell necroses. Calcification of the Tamm–Horsfall protein in the distal tubule may also arise. Most significantly, there are no extended areas of tubular necrosis, as is characteristic for heavy-metal toxicity (such as with mercury, platinum, cadmium or uranium) or aminoglycoside toxicity (such as with gentamicin) (Fig. 4).

The least frequent changes with CyA treatment are those of vasculopathy, which are seen almost only in the afferent arteriole. These changes consist of injury to the endothelium and smooth muscle cells in the vessel wall which can lead to vessel occlusion and, ultimately, to its obliteration. This, in turn, results in a localized ischaemia with glomerular collapse initially, followed later by glomerulosclerosis. There is an associated development of tubular atrophy within regions of interstitial fibrosis, which assumes a striped form and is possibly a reflection of the loss of blood supply to the tissue (Fig. 4).

REVERSIBILITY

Functional alterations to the tubular system are fully reversible. Serum magnesium, potassium and uric acid return to normal in both patients and in animals on discontinuation of the drug. Similarly, functional changes to the vascular system are fully reversible, and the signs of renal vasoconstriction and dysfunction disappear when CyA is withdrawn. These changes need not be associated with vascular-interstitial damage in patients and, in animals, such structural changes do not arise.

Structural alterations to the tubular system are also generally reversible. The isometric vacuoles and giant mitochondria disappear, and the single necrotic cells are replaced when therapy is discontinued, although the microcalcification of the Tamm–Horsfall protein may persist. In contrast, structural vascular changes with their associated tubulointerstitial damage are not reversible, once established. Although mild vessel wall damage is reparable to some extent, vessel occlusion and glomerulosclerosis persist, and the striped interstitial fibrosis with tubular atrophy is maintained.

RELEVANCE OF ALTERATIONS

Alterations to tubular function are of little concern because the changes in serum magnesium, potassium or uric acid, although common, are minor, not associated with decreases in sodium reabsorption or urinary concentrating power, and are reversible. Similarly, alterations to tubular structure are no longer common, are not associated with extensive tubular necrosis and are reversible. Thus, tubular changes (Fig. 5) appear to have little clinical relevance.

In contrast, the changes of renal perfusion and glomerular filtration are frequent and, although reversible in principle, precede and predispose to irreversible structural alterations. Without doubt, these vascular changes and their associated interstitial fibrosis and tubular atrophy are important because, although rare, they are permanent. Thus, both types of vascular alteration (Fig. 6) are clinically relevant and have considerable significance for patient safety.

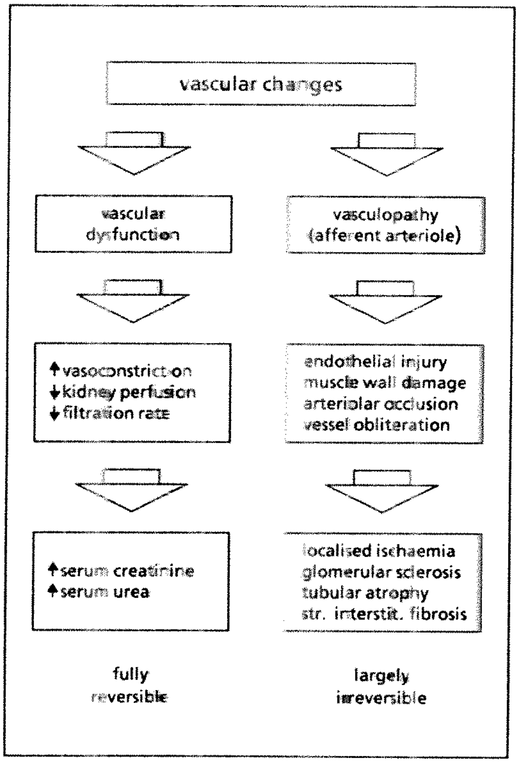

FIGURE 6. Vascular changes with CyA.

PREDICTABILITY OF RENAL DAMAGE

The two events that are most clinically relevant—vascular dysfunction, expressed as vasoconstriction, and vasculopathy, associated with interstitial fibrosis and tubular atrophy—have not been proven to be causally related. However, it has been established that the magnitude of vascular dysfunction, reflected by the rise in serum creatinine, is related to the incidence of vasculopathy. Thus, by avoiding severe vascular dysfunction, it is possible to avoid severe structural damage to the kidney.

This is because the development of structural changes to the vessels is strongly dose-dependent, occurs at high doses and is associated with considerable changes in function. The occurrence of functional changes to the vessels is also dose-dependent, but occurs at low doses at which structural changes do not develop. Hence, restricting the dose while limiting the functional changes will avoid the simultaneous occurrence of inappropriately high doses and severe functional disturbances.

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Indirect assessment of renal dysfunction in patients taking cyclosporin A for autoimmune diseases

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SUMMARY

The reliability of increases in serum creatinine and serum urea above initial values in detecting decreases in glomerular filtration rate below initial values was assessed in patients taking cyclosporin A (CyA) for autoimmune diseases. Both serum creatinine and serum urea provided reasonable estimates of the decline in glomerular filtration rate when measured simultaneously and when compared to patients' own baseline values. Combination of both serum creatinine and serum urea improved the assessment of renal dysfunction, partly by reducing the influence of any analytical errors. Frequent measurements, precise baseline evaluation and standardization of procedures can be expected to increase further the precision of assessing renal dysfunction from serum measurements. Hence, careful evaluation of changes in serum creatinine, with the option to include changes in serum urea, can provide a simple and reliable way of monitoring changes in renal function in patients taking CyA for autoimmune diseases.

In patients receiving prolonged treatment with cyclosporin A (CyA) for autoimmune diseases, the potentiality for developing permanent damage to the kidney is of major concern. The incidence of such changes is steadily decreasing as a result of an increasing awareness of the factors that predispose towards its development. Nevertheless, to avoid the major risk and maintain patient safety, renal function must be carefully followed during therapy to control renal dysfunction.

There are many problems in measuring changes in renal function directly. Inulin clearance is considered the 'golden standard', but inulin is not available in all countries and the method is often considered too cumbersome for regular use.[1] Other marker substances are generally less suitable both for theoretical reasons[1] and for practical reasons when they are radiolabelled. Also, the use of clearance from plasma disappearance curves, rather than from urine collections, is known to introduce inaccuracies.[1]

Indirect assessments of renal function, such as monitoring of serum chemistry, are also

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problematical. Serum creatinine is an imperfect indicator of decreases in renal function because of tubular secretion.[1] Because the degree of secretion rises as renal function declines, the increase in serum creatinine can underestimate the degree of renal dysfunction.[1,2] Similarly, serum urea gives imprecise estimates of decreases in renal function because of tubular reabsorption and a dependence on diet.[1] Since the degree of reabsorption rises as tubular flow decreases, the increase in serum urea can overestimate any decline in renal function.

Although creatinine and urea are clearly unsuitable for assessing renal function accurately, they may be adequate for estimating the degree of renal dysfunction, if analysed and interpreted with appropriate care. Certainly, if no direct methods of measuring renal function are available, these have to suffice, despite their limitations. Hence, an objective evaluation of their reliability in predicting known decreases in renal function needs to be made. In patients treated with CyA for most autoimmune diseases especially, there is generally no underlying renal disease and renal dysfunction is modest, so that neither creatinine secretion nor urea reabsorption would be expected to increase much above normal.

PATIENTS

The patients in this study suffered from psoriasis, rheumatoid arthritis, Crohn's disease or juvenile diabetes of recent onset and were treated with CyA for control of their disease. Although an indirect assessment of renal dysfunction by monitoring serum creatinine was mandatory, another indirect method—monitoring serum urea—was often included. Because of a concern for safety, many investigators used a direct method to measure renal function and determined glomerular filtration rate in addition.

The patients selected for analysis were monitored before and at least once during therapy. In each patient, glomerular filtration rate and two other parameters were determined simultaneously. When assessments were made at intervals during therapy, the one displaying the largest decrease in glomerular filtration rate was selected. Thus, three methods of assessing renal function were evaluated at the same time during therapy and each of these could be compared to the patient's own control value measured before therapy.

THEORETICAL CONSIDERATIONS

Both serum creatinine and urea are inversely related to glomerular filtration rate and both rise as renal function declines. Proportionality constants can be defined to relate glomerular filtration rate to the reciprocal value of serum creatinine or urea. These constants include the amount of creatinine secreted into the tubules and the extent of urea reabsorption from the tubular system. These processes cause the amount of creatinine or urea in the urine to differ from the amount filtered at the glomerulus.

It is possible that the constants that describe this non-ideal behaviour of creatinine and urea are not the same before the start of CyA therapy as during therapy, when renal function is depressed. For the purposes of data analysis, however, it was assumed that these constants were uninfluenced by therapy. Then it was determined to what extent the predictions based on this assumption were, in fact, true.

DATA ANALYSIS

The relationship between serum creatinine and glomerular filtration rate at baseline and during therapy with CyA is expressed by: $GFR_0 = a_0 \times 1/Cr_0$ and $GFR_x = a_x \times 1/Cr_x$. In these

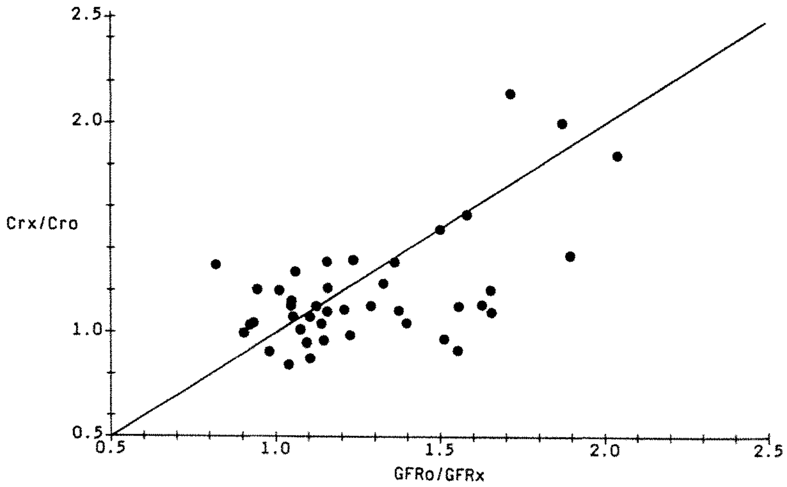

FIGURE 1. Correlation between glomerular filtration and serum creatinine ratios.

equations, a_o and a_x include the secretion of creatinine into the tubules and reflect the increase in creatinine excretion in comparison to the amount filtered. Thus, by dividing, $GFR_o/GFR_x = a_o/a_x \times Cr_x/Cr_o$. If, however, a_o equals a_x because creatinine secretion did not change during CyA therapy, then $GRF_o/GFR_x = Cr_x/Cr_o$.

The relationship between serum urea and glomerular filtration rate at baseline and during therapy with CyA is expressed by: $GFR_o = b_o \times 1/Ur_o$ and $GFR_x = b_x \times 1/Ur_x$. In these equations, b_o and b_x include the tubular reabsorption of urea and reflect the decrease in excretion of urea compared to the amount filtered. Thus, by dividing, $GFR_o/GFR_x = b_o/b_x \times Ur_x/Ur_o$. If, however, b_o equals b_x because urea reabsorption did not change during CyA therapy, then $GFR_o/GFR_x = Ur_x/Ur_o$.

RESULTS

When the glomerular filtration ratio (1·28 ± 0·05, mean ± SEM) was compared with the inverse creatinine ratio (1·19 ± 0·04, mean ± SEM), the paired difference between the two was 0·08 ± 0·04 (mean ± SEM), indicating only a minor increase in creatinine secretion. When the glomerular filtration ratio was plotted against the inverse creatinine ratio (Fig. I), the relationship appeared to be linear ($r = 0·587$) and the points were distributed relatively evenly around the line of identity.

When the glomerular filtration ratio (1·28 ± 0·05, mean ± SEM) was compared with the inverse urea ratio (1·42 ± 0·06, mean ± SEM), the paired difference between the two was 0·14 ± 0·05 (mean ± SEM), suggesting that urea reabsorption had increased only slightly during CyA therapy. When the glomerular filtration ratio was plotted against the inverse urea ratio (Fig. 2), the relationship appeared to be linear ($r = 0·633$) and the points were distributed only slightly above the line of identity.

Thus, as expected, the glomerular filtration ratio is only marginally underestimated by the inverse creatinine ratio, and is only slightly overestimated by the inverse urea ratio when the decrease in glomerular filtration is modest. By combining both ratios of creatinine and urea it should therefore be possible to allow the one to counterbalance the other and also to reduce the influence of analytical errors in either determination.

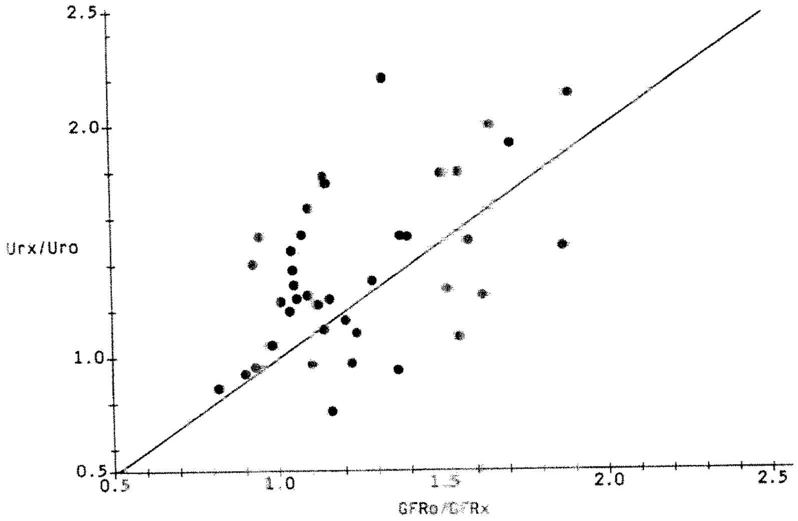

FIGURE 2. Correlation between glomerular filtration and serum urea ratios.

When the glomerular filtration ratio (1·28 ± 0·05, mean ± SEM) was compared with the average of both individual creatinine and urea ratios (1·31 ± 0·05, mean ± SEM), the two estimates differed by almost nothing (0·02 ± 0·05, mean ± SEM). When the glomerular filtration ratio was plotted against the average creatinine and urea ratio (Fig. 3), the relationship appeared to be more linear (r = 0·722) and the points were distributed symmetrically around the line of identity.

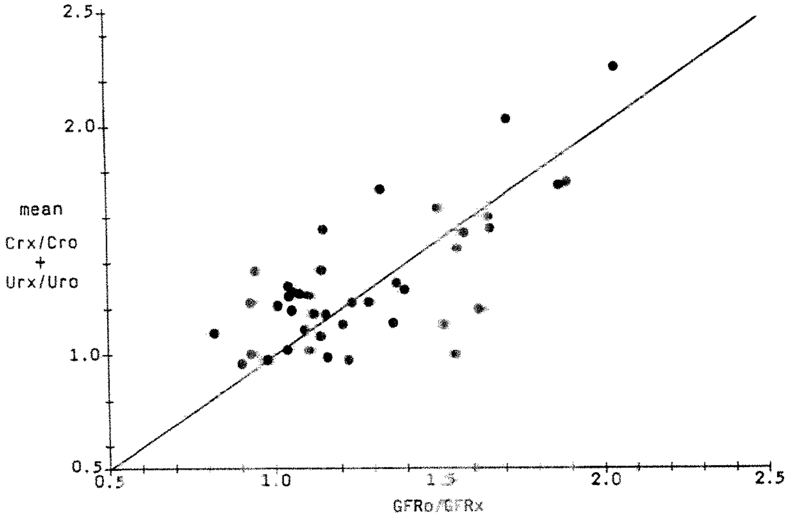

FIGURE 3. Relationship between glomerular filtration ratio and mean of serum creatinine and serum urea ratios.

TABLE I. Corresponding changes in serum creatinine (Cr) and glomerular filtration rate (GFR) with CyA

ΔCr	Cr_x/Cr_o	GFR_x/GFR_o	ΔGFR
+33%	1·33	0·75 (1/1·33)	−25%
+50%	1·50	0·66 (1/1·50)	−33%
+66%	1·66	0·60 (1/1·66)	−40%
+100%	2·00	0·50 (1/2·00)	−50%

DISCUSSION

Indirect assessment of renal function using serum creatinine can estimate the degree of renal dysfunction with adequate precision when compared with the patient's own starting value. As individual determinations vary considerably, measurements should be repeated to distinguish stable from slowly declining renal function. This procedure, rather than direct measurements of renal function using clearance techniques, is always used for routine monitoring following kidney transplantation.

Because the decrease in renal function is modest when treating autoimmune diseases with low-dose CyA, it is essential to establish a reliable baseline. By confirming that two or more assessments are in agreement before starting treatment, the error resulting from a single, incorrect starting value can be avoided. By ensuring that measurements are obtained under standardized conditions, such as in the morning, while fasted and before CyA dosing, even small changes in renal function can be detected.

The use of a second parameter to increase the reliability of the monitoring can improve the assessment of renal dysfunction by identifying incorrect determinations. Serum urea is particularly convenient for this purpose as it is easily measured at the same time as serum creatinine. If serum urea but not serum creatinine is elevated, the patient is probably dehydrated, has increased protein intake or there is an analytical error. However, if serum creatinine but not serum urea is raised, the creatinine determination is probably incorrect and should be repeated.

Considerable care is needed to contain renal dysfunction within reasonable limits when a direct assessment of renal function is available. In theory, the relationship between glomerular filtration rate and serum creatinine is inverse, not direct, and the present analysis confirms that this theoretical relationship is retained during CyA treatment. As a result, the percentage decrease in glomerular filtration rate is always less than the corresponding increase in serum creatinine. Thus, it is potentially dangerous to allow filtration rate to decline by the same percentage that creatinine is normally allowed to rise. Every percentage decrease in filtration rate is associated with a larger percentage increase in serum creatinine (Table I).

All current assessments of the risk of developing permanent structural damage to the kidney are based on indirect assessments of renal dysfunction, as expressed by the increase in serum creatinine above the patient's own baseline value. By restricting the dose to less than 5 mg/kg/day and limiting renal dysfunction so that serum creatinine rises no more than 30% above baseline, this risk is virtually eliminated. Whereas the rise in serum urea can also predict the likelihood of developing permanent kidney damage, this risk cannot be assessed from the decrease in glomerular filtration rate because insufficient measurements are available in biopsied patients. Consequently, the only *certain* way to ensure the safety of the patient is to prevent serum creatinine from rising to the level known to be associated with renal damage.

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Predictive value of cyclosporin A level for efficacy or renal dysfunction in psoriasis

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SUMMARY

Cyclosporin A (CyA) trough (pre-dose) levels were measured in whole blood with a radioimmunoassy (RIA) using specific monoclonal antibody in 193 patients receiving CyA for the treatment of psoriasis. These patients received either CyA 2·5 mg/kg/day ($n = 134$) or 5 mg/kg/day ($n = 59$). In addition, a subgroup of 94 patients also had CyA trough levels measured using a non-specific polyclonal RIA. Within each CyA dose group, no difference was detected between mean CyA trough levels in relation to success or failure nor to the presence or absence of renal dysfunction with the use of either the specific or non-specific RIA. Based on the experience in transplantation, CyA thresholds of 100 and 200 ng/ml (specific) were selected for the assessment of efficacy and renal dysfunction. The success rate was higher by 10–15% when the CyA level was above, rather than below, 100 ng/ml in both the CyA 2·5 mg and 5 mg/kg/day groups. A slightly increased incidence of renal dysfunction was only found in the 5 mg/kg/day group when the CyA level was above 200 ng/ml. Because of its low predictive value, measurement of the level of CyA was not particularly useful for monitoring patients with psoriasis treated with low-dose CyA.

Cyclosporin A (CyA) blood levels have been used for routine monitoring of CyA-treated transplant recipients since early in its clinical use. The objective was to achieve concentrations consistent with clinical efficacy, but which did not give rise to unwanted side-effects. Although the usefulness of CyA blood trough level monitoring in transplantation has been demonstrated, the therapeutic window needed to be defined according to the indication and the time after transplantation.[1-4]

The conditions of CyA treatment of psoriasis are very different from those of transplantation. Low-dose CyA has proved to be effective in treating severe psoriasis and has been associated with very limited renal dysfunction.[5,6] Therefore, it was of interest to evaluate the clinical value of CyA trough-level monitoring for assessing the efficacy and safety of the drug specifically in psoriatic patients.

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PATIENTS AND METHODS

One-hundred and ninety-three patients were included in the analysis. These patients had participated in two multicentre European studies and were treated in 30 hospital dermatology departments. There were 133 males and 60 females; all were adults (mean ± SEM age: 42 ± 0·7 years) who had had severe, disabling chronic plaque-type psoriasis for a mean duration of 17 ± 0·5 years. Previous therapies for the psoriasis had consisted of retinoids in 55% and methotrexate in 28%. The mean cumulative dose of methotrexate was 5·8 ± 1·8 g. One-hundred and thirty-four patients received CyA 2·5 mg/kg/day and 59 received 5 mg/kg/day, divided into two daily doses.

In all 193 patients, the concentration of CyA was measured in whole blood using a radioimmunoassay (RIA) with a monoclonal antibody specific to the parent compound and a tritium-labelled tracer (Sandimmun-kit®, Sandoz Pharma).[7–9] A subgroup of 94 patients also had CyA levels measured by RIA, using a non-specific polyclonal antiserum which partially cross-reacted with metabolites (Ciclo-kit®, Sandoz Pharma).[10] A pre-dose blood sample was taken at 12 hours (trough level) after drug intake

The first 4 weeks of treatment were selected for analysis as the CyA dose remained unchanged during this period of time. Later, dose changes were prescribed according to protocol, based on efficacy, renal dysfunction or on the blood levels themselves. The improvement in psoriasis and the incidence of renal dysfunction at Week 4 was sufficient to make an evaluation at that time worthwhile. For each patient, the mean value of four blood level measurements (taken at the end of Weeks 1, 2, 3 and 4) was calculated and correlated to efficacy and renal dysfunction at the end of Week 4. Only the patients for whom four consecutive blood level measurements were available were selected for analysis. Clinical efficacy was measured by the changes in psoriasis severity as assessed by the Psoriasis Area and Severity Index (PASI) score.[11] Success was defined as a PASI score reduction greater than or equal to 75%, partial success by a PASI score reduction of 50–74%, and insufficient efficacy by a PASI score reduction of less than 50%. Renal dysfunction was defined as an increase in serum creatinine greater than or equal to 30% above baseline level, measured before starting CyA treatment, or between 20% and 29%.[12]

Results were reported as means ± SEM. Comparison of the mean values was carried out using Student's *t*-test. The incidence of success or renal dysfunction was compared between the groups using Fisher's exact test.

RESULTS

The mean specific CyA blood trough level was 103 ± 5 ng/ml (± SEM) with CyA 2·5 mg/kg/day and 230 ± 17 ng/ml, with 5 mg/kg/day. Non-specific mean levels were 289 ± 16 ng/ml and 585 ± 45 ng/ml respectively, corresponding to a specific:non-specific ratio of 0·37. No significant difference was found between the levels measured at Weeks 1, 2, 3 or 4. Previous therapies with methotrexate or retinoids did not result in significant differences in CyA blood trough levels (Table I).

Mean PASI score reduction after 4 weeks of treatment was 39 ± 3% with CyA 2·5 mg/kg/day and 67 ± 3% with 5 mg/kg/day. Creatinine percentage increases over baseline during the same time period were 6 ± 1% and 10 ± 2%, respectively.

TABLE 1 Specific CyA blood trough levels (ng/ml) according to previous therapies for psoriasis*

	Pre-treatment	No pre-treatment
Retinoids†		
CyA 2·5 mg/kg/day	(*n* = 77) 99 ± 7	(*n* = 56) 107 ± 9
CyA 5 mg/kg/day	(*n* = 35) 229 ± 20	(*n* = 21) 236 ± 34
Methotrexate‡		
CyA 2·5 mg/kg/day	(*n* = 31) 113 ± 13	(*n* = 99) 100 ± 6
CyA 5 mg/kg/day	(*n* = 15) 255 ± 36	(*n* = 31) 212 ± 25

*Monoclonal RIA.
†Pre-treatment data missing for four patients.
‡Pre-treatment data missing for 17 patients.
Figures presented are means ± SEM of Weeks 1, 2, 3 and 4.

Efficacy

No correlation was found between the percentage of PASI score reduction and specific or non-specific blood levels in either CyA 2·5 mg or 5 mg/kg/day groups (Fig. I). Furthermore, in both of these CyA treatment groups, mean specific levels were not significantly different in patients with clinical success compared with those who had partial success or insufficient

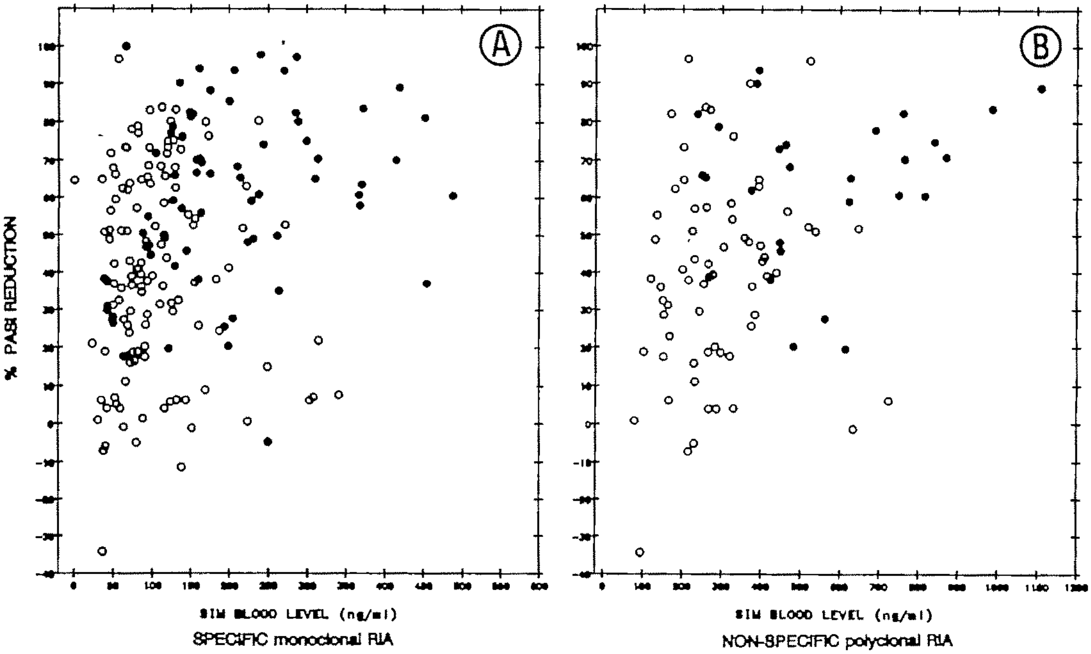

FIGURE 1. Percentage reduction of PASI (Psoriasis Area and Severity Index) measured after 4 weeks of treatment according to SIM (cyclosporin A; CyA) blood trough levels (ng/ml) as measured by specific monoclonal (A) or non-specific polyclonal (B) RIA. Open circle: CyA 2·5 mg/kg/day. Solid circle: CyA 5 mg/kg/day. Each circle represents one patient and is the mean value of four CyA blood levels taken at Weeks 1, 2, 3 and 4.

TABLE 2. Cyclosporin A blood trough levels (ng/ml) according to efficacy* after 4 weeks of treatment

	Insufficient efficacy (PASI reduction < 50%)	Partial success (PASI reduction 50–74%)	Success (PASI reduction ≥ 75%)
CyA 2·5 mg/kg/day			
Specific level	(*n* = 83) 100 ± 7	(*n* = 38) 100 ± 9	(*n* = 15) 125 ± 12
Non-specific level	(*n* = 46) 272 ± 19	(*n* = 17) 326 ± 35	(*n* = 7) 304 ± 44
CyA 5 mg/kg/day			
Specific level	(*n* = 15) 198 ± 24	(*n* = 24) 224 ± 23	(*n* = 20) 260 ± 37
Non-specific level	(*n* = 6) 496 ± 31	(*n* = 10) 619 ± 55	(*n* = 9) 632 ± 106

*According to reductions in Psoriasis Area and Severity Index (PASI).
Specific = monoclonal RIA; non-specific = polyclonal RIA.
All figures are means ± SEM of Weeks 1, 2, 3 and 4.

efficacy (Table 2). This was also true for the non-specific CyA level measurements. Interestingly, specific as well as non-specific CyA levels were significantly higher ($P = 0·02$) in patients with insufficient efficacy while receiving CyA 5 mg/kg/day than in patients with success while taking 2·5 mg/kg/day.

On the basis of experience in maintenance therapy for transplantation,[3,13,14] a threshold level of 100 ng/ml (specific measurement) was selected for analysing the success rate in relation to the blood trough level. The non-specific threshold level of 275 ng/ml was subsequently derived from the specific level by applying the above-mentioned specific:non-specific ratio. Results are summarized in Table 3. The success rate was slightly higher when the specific level was greater than or equal to 100 ng/ml than when it was below this level: 19% *vs* 6% with CyA 2·5 mg/kg/day ($P = 0·027$, Fisher's exact test); and 35% *vs* 20% with 5 mg/kg/day (not significant). However, within each group, a wide interindividual variation of blood level was observed despite the stable dose of CyA (Fig. 1), a clear illustration of its low predictive value for efficacy. No difference in the remission rate was found on assessing the non-specific levels (Table 2).

Renal dysfunction

No correlation was detected between the percentage change in serum creatinine (compared with baseline) and the specific as well as non-specific CyA blood trough levels in either CyA 2·5 mg or 5 mg/kg/day groups (Fig. 2). The decreases of creatinine detected in some patients (negative values) were mainly due to spontaneous variations in creatinine measurements.[12]

The mean CyA blood level did not differ whether the creatinine increase was below 20%, between 20% and 29%, or equal to or greater than 30% after 4 weeks of treatment in both the CyA 2·5 mg and 5 mg/kg/day groups (Table 4).

Based on the experience in transplantation,[3,13,14] threshold levels of 200 ng/ml (specific) or 550 ng/ml (non-specific) were selected for investigating the incidence of renal dysfunction. Results are summarized in Table 5. The incidence of renal dysfunction was slightly increased according to whether the specific level was below or above 200 ng/ml in both the CyA 2·5 mg and 5 mg/kg/day groups: 10% *vs* 6% with 2·5 mg/kg/day (not significant); 17% *vs* 0% with 5 mg/kg/day ($P = 0·049$, Fisher's exact test). No significant difference was found with the

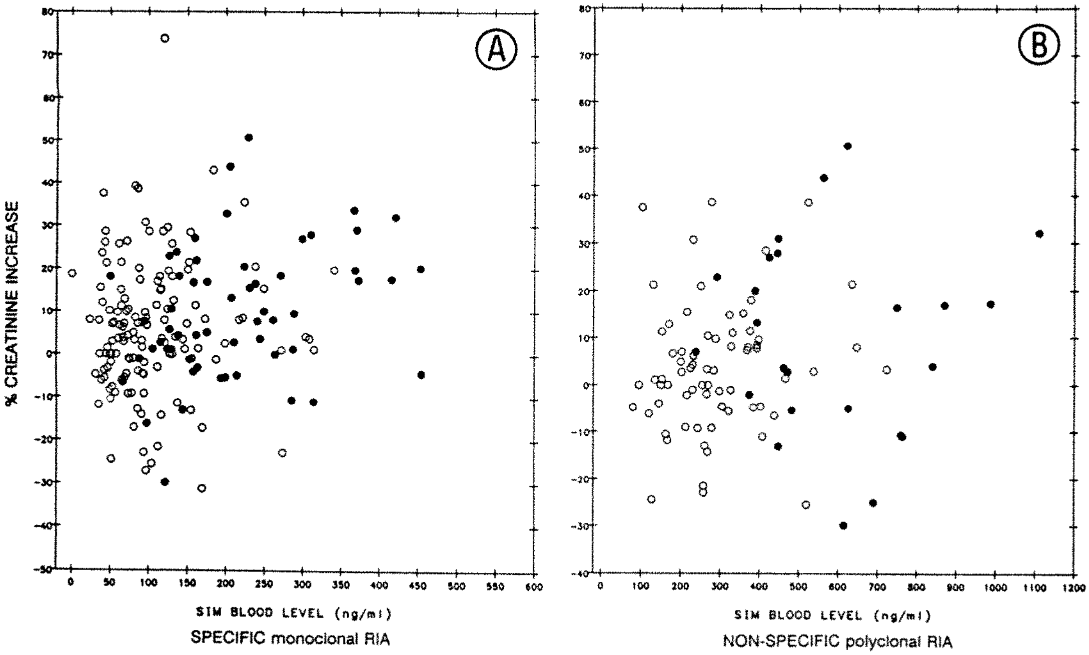

FIGURE 2. Percentage change in serum creatinine (over baseline values) after 4 weeks of treatment according to SIM (cyclosporin A; CyA) blood trough levels (ng/ml) as measured by specific monoclonal (A) or non-specific polyclonal (B) RIA. Open circle: CyA 2·5 mg/kg/day. Solid circle: CyA 5 mg/kg/day. Each circle represents one patient and is the mean value of four CyA blood levels taken at Weeks 1, 2, 3 and 4.

non-specific measurements in either the CyA 5 mg/kg/day (25% *vs* 8%) or 2·5 mg/kg/day (0% *vs* 6%) group.

However, the wide interindividual variability of CyA blood levels resulted in their low predictive value for renal dysfunction (Fig. 3); indeed, renal dysfunction occurred in some patients despite low CyA levels.

DISCUSSION

The efficacy of CyA in psoriasis[5,6] has been firmly established with a dose of 5 mg/kg/day. Furthermore, a clear dose–response relationship has been demonstrated with lower doses. Significant improvement with CyA in comparison to placebo was detected as early as 1 month after starting treatment. However, the maximum response rate was observed at 3 months, when the success rate was nearly 50% with CyA 2·5 mg/kg/day and 90% with 5 mg/kg/day. In psoriasis, the rapidity of the clinical response permitted the evaluation of the predictive value of blood levels after a short period of time when CyA dosages were stable.

No significant differences were found in the mean values of CyA whole blood trough levels in terms of clinical outcome or renal dysfunction, whether measurement was specific or non-specific. There may be several explanations for these disturbing results. The first is the well known inter- and intraindividual variability of CyA levels.[3,15] In the present analysis, intraindividual variability was minimized by using the mean value of at least four consecutive level measurements for each patient. On the other hand, these patients were from multicentre,

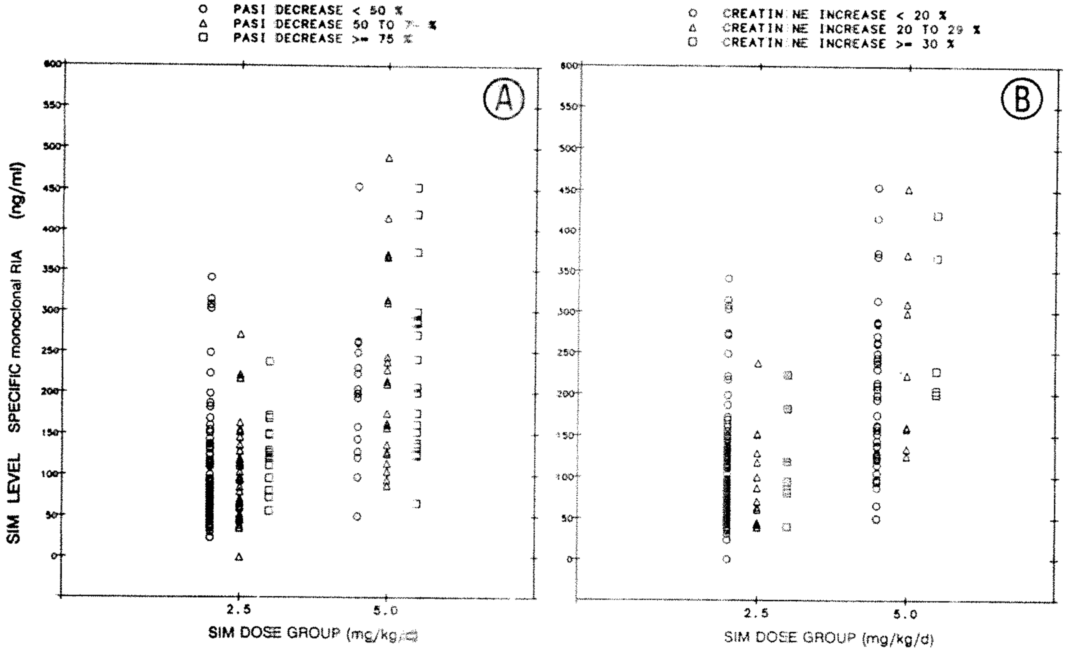

FIGURE 3. Distribution of specific SIM (cyclosporin A; CyA) blood trough levels (monoclonal RIA) according to efficacy (A) or renal dysfunction (B) after 4 weeks of treatment with CyA 2·5 mg or 5 mg/kg/day. Each symbol represents one patient and is the mean value of 4 CyA blood levels taken at Weeks 1, 2, 3 and 4.

multilaboratory trials and, despite the participation of most of the laboratories in a CyA quality assessment scheme, interlaboratory as well as interindividual variability was likely.[16] The second explanation is that evaluation of only the mean values does not take into account a threshold effect, especially if the overall incidence of the effect is low. Another explanation, which applies to the CyA 2·5 mg/kg/day group, is that the CyA trough level was close to the

TABLE 3. Success* rates after 4 weeks of treatment according to CyA blood trough levels

	CyA (mg/kg/day)		
	2·5	5	Total
Specific CyA level			
< 100 ng/ml	(5/81) 6%	(1/5) 20%	(6/87) 7%
≥ 100 ng/ml	(10/53) 19%	(19/54) 35%	(29/107) 27%
Non-specific CyA level			
< 275 ng/ml	(4/38) 11%	(1/1) –	(5/39) 13%
≥ 275 ng/ml	(3/33) 9%	(8/23) 35%	(11/56) 20%

*Success = ≥ 75% reduction of PASI score.
Specific = monoclonal RIA; non-specific = polyclonal RIA (mean of Weeks 1, 2, 3 and 4).
(Number of patients/total).

TABLE 4. Cyclosporin A blood trough levels (ng/ml) in relation to creatinine increase after 4 weeks of treatment

	Creatinine increase above baseline		
	< 20%	20–29%	≥ 30%
CyA 2·5 mg/kg/day			
Specific level	(n = 113) 104 ± 6	(n = 15) 98 ± 14	(n = 7) 118 ± 24
Non-specific level	(n = 61) 285 ± 16	(n = 4) 357 ± 109	(n = 4) 283 ± 88
CyA 5 mg/kg/day			
Specific level	(n = 44) 214 ± 20	(n = 9) 248 ± 39	(n = 5) 284 ± 46
Non-specific level	(n = 16) 610 ± 52	(n = 4) 386 ± 34	(n = 4) 684 ± 146

Specific = monoclonal RIA; non-specific = polyclonal RIA.
All figures are means ± SEM of Weeks 1, 2, 3 and 4.

limit of sensitivity of the method. However, despite these reservations, such results reflect the clinical value of routine CyA blood trough level monitoring on a large scale.

A significant relationship was found between both a specific CyA blood trough level of less than 100 ng/ml and inefficacy, and a level greater than or equal to 200 ng/ml and renal dysfunction. However, the predictive value was low; for example, with CyA 2·5 mg/kg/day, the overall success rate after 1 month was 11%. In the subset of patients whose CyA blood levels were greater than or equal to 100 ng/ml, the success rate was 19%. This low predictive value has to be viewed in comparison to the clinical characteristics of CyA treatment of psoriasis. First, the therapeutic response is rapid, within the first month and most often within the first 2 weeks.[5,6,17] Second, the clinical evaluation of efficacy is extremely easy to carry out and, third, an early failure may very well respond to a dosage increase later. For safety monitoring, the overall incidence of CyA-related renal dysfunction on a low dose of the drug should be considered first. This incidence was only 6% in this series of patients after 1 month of treatment with CyA 2·5 mg/kg/day. Also, the extent of creatinine increase was very limited (+6·1%, corresponding to a mean concentration of 85 μmol/l). Finally, renal dysfunction does

TABLE 5. Percent of patients with relevant renal dysfunction* after 4 weeks of treatment according to CyA blood trough level

	CyA group (mg/kg/day)		
	2.5	5	Total
Specific level			
< 200 ng/ml	(7/124) 6%	(0/23) 0%	(7/152) 5%
≥ 200 ng/ml	(1/10) 10%	(5/30) 17%	(6/40) 15%
Non-specific level			
< 550 ng/ml	(4/66) 6%	(1/12) 8%	(5/78) 6%
≥ 550 ng/ml	(0/13) 0%	(3/12) 25%	(3/25) 20%

*Creatinine increase at Week 4 ≥ 30% above baseline.
Specific = monoclonal RIA; non-specific = polyclonal RIA (mean of Weeks 1, 2, 3 and 4).
(Number of patients/total).

not occur suddenly with a low dose of CyA and, thus, allows adaptation of the dose according to the results of the serum creatinine.[12] These features and the low positive predictive value of CyA blood levels for efficacy as well as renal dysfunction do not favour the routine monitoring in patients treated with low doses of the drug.

There was a trend for improved predictive values for efficacy with specific, rather than non-specific, levels. When considering the CyA 2·5 mg and 5 mg/kg/day groups together, the difference between success rates according to specific drug levels above or below 100 ng/ml was 20%; for non-specific levels (threshold level 275 ng/ml), the difference was 7%. For renal dysfunction, however, results obtained with specific or non-specific levels were similar; there was a 10% difference in the incidence of renal dysfunction in relation to a specific level above or below 200 ng/ml, and a 14% difference in relation to a non-specific level (threshold level 550 ng/ml).

Finally, interindividual variations of sensitivity to CyA, for efficacy as well as for renal dysfunction, appears to be much more important than the therapeutic gain to be expected from careful pharmacological monitoring. Routine drug level monitoring does not appear to be helpful in the treatment of patients with psoriasis. There is, however, no question as to the use of the measurement of the level of CyA in unusual conditions such as drug interactions.[18]

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Kidney biopsies in control or cyclosporin A-treated psoriatic patients

INTERNATIONAL KIDNEY BIOPSY REGISTRY OF CYCLOSPORIN A (SANDIMMUN®) IN AUTOIMMUNE DISEASES*

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SUMMARY

Kidney biopsies were obtained from 14 adult patients treated with cyclosporin A (CyA) for psoriasis. These biopsies were compared with those from 16 psoriatic patients of the same age, but who were not receiving treatment with CyA. Patients receiving CyA had a maximum mean dose of 5·1 mg/kg/day for a mean duration of 15 months (range 3–37 months). Minimal or slight interstitial fibrosis, tubular atrophy or arteriolopathy were seen in the biopsies of patients receiving CyA as well as in those of the controls. No relevant CyA-related structural alterations of the kidney were detected.

Cyclosporin A (CyA) is an effective therapy for severe psoriasis at doses of 2.5 mg–5 mg/kg/day.[1,2] However, as relapses occur after termination of CyA administration,[3] continuous therapy is probably necessary over a long period of time (1 year or more) and the safety of CyA treatment must be carefully defined.

The main concern with the use of CyA is the risk of renal impairment. Changes in renal function, as reflected by the level of the serum creatinine, were limited in those patients treated with low-dose CyA (2·5 mg–5 mg/kg/day) over a long period of time.[4] In contrast, CyA-associated irreversible structural alterations of the kidney were reported with high doses of CyA in transplant recipients as well as in patients treated for autoimmune diseases.[5-7] Cyclosporin A nephropathy was described as a combination of arteriolopathy, interstitial fibrosis ('striped' form) and focal tubular atrophy. The arteriolopathy is characterized by nodular protein deposits in the media which replace necrotic myocytes in the arteriolar wall and may narrow the vascular lumen. Mucoid thickening of the intima may also be seen. Irregular foci or stripes of interstitial fibrosis with atrophic tubules in the absence of significant interstitial inflammation may be observed in the cortex.[8]

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In autoimmune diseases, these findings were only reported in patients treated with a CyA dose of more than 7 mg/kg/day. In these patients, the CyA dose and extent of creatinine increase were found to be major additive risk factors.[9] It was, therefore, of importance to assess the morphology of the kidney biopsies in psoriatic patients receiving low-dose treatment with CyA.

PATIENTS AND METHODS

Patients

Thirty kidney biopsies were obtained from 25 adult patients who had severe chronic plaque psoriasis, 14 of which were performed during or after CyA treatment and 16 of which were obtained before receiving CyA. Of these 25 patients, five (patients 10–14) had a biopsy taken both before and after CyA treatment.

The group receiving CyA included eight males and six females whose mean age was 40 years (range 21–64 years). The initial mean CyA dose was 3·3 mg/kg/day (range 1·2–5·5 mg). Only three patients were still taking CyA at the time of biopsy. The drug was stopped in the remaining patients for a mean duration of 1·8 months (range 0·5–4 months) before biopsy. None of the patients had received non-steroidal anti-inflammatory agents.

The nine males and seven females whose biopsies were taken before starting CyA therapy had a mean age of 43 years (range 21–64 years). All patients had a serum creatinine concentration within the normal limits at the time of entry into the trial or at biopsy. Serum creatinine was measured every 1–2 weeks during the first 2 months of treatment and every 4–6 weeks thereafter.

Kidney biopsies

All except two biopsies were analysed by the local pathologist and the review pathologist who was not aware of the treatment received by the patients. On the basis of light microscopy examinations, the following data were recorded: number of glomeruli; number of obsolescent glomeruli; and number of glomeruli with segmental focal glomerulosclerosis. Arteriolopathy, interstitial fibrosis and tubular atrophy were scored as: none = 0; minimal = 1; slight = 2; moderate = 3; and severe = 4.

In most cases, the decision for biopsy during CyA treatment was to ensure the safety of continuation of CyA beyond the end of the clinical trial (1 year) in patients who experienced medical benefit from the treatment. For some, however, obtaining a kidney biopsy after 1 year of CyA treatment was included in the protocol. All protocols (including the 16 biopsies performed before CyA treatment) were approved by the various Ethics Committees involved.

RESULTS

Cyclosporin A dose and serum creatinine concentration

The initial mean CyA dose was 3·3 mg/kg/day (range 1·2–5·5 mg). The maximum dose was 5·1 mg/kg/day (mean range 2·8–7·7) and the mean dose up to the time of biopsy was 3·9 mg/kg/day (range 2·6–5·2) (Table I). The maximum CyA dose was below 6 mg/kg/day in 13 of the 14 patients, although one patient received 7·7 mg/kg/day for less than 1 month (in this case, the mean CyA dose up to biopsy was 5·2 mg/kg/day). The mean duration of CyA treatment at the time of biopsy was 15 months (range 3–37 months) (Table I).

TABLE 1. Baseline and treatment characteristics of CyA-treated psoriatic patients with kidney biopsy

Patient no.	Age (years)	Gender	Duration of CyA treatment (mos)	CyA dose (mg/kg/day)		Creatinine increase	
				Max	Mean up to biopsy	Max	Last with CyA
01	64	F	37	5.0	3.5	71%	71%*
02	44	F	15	2.8	2.6	18%	18%
03	54	M	16	5.2	4.3	22%	8%
04	33	M	16	5.0	4.0	44%	3%
05	29	M	17	5.1	4.5	58%	13%
06	21	F	13	5.8	4.3	5%	−33%
07	27	M	15	5.6	4.8	21%	−17%
08	28	M	17	4.8	2.9	25%	19%
09	46	M	15	5.5	3.9	52%	−6%
10	39	F	5	5.4	4.7	6%	−8%
11	20	F	14	5.7	3.7	99%	—
12	42	F	3	3.1	2.6	28%	—
13	57	M	11	5.1	3.1	39%	24%*
14	38	M	13	7.7	5.2	96%	20%*

*Still receiving CyA.

With CyA, the maximum serum creatinine increase over the baseline value was 42% (range 5–99%). At the time of biopsy, it was −4% (range −33–19%) in patients who had stopped CyA before biopsy, and 20%, 24% and 71% in the three patients who were still taking CyA at the time of biopsy. A serum creatinine increase of more than 50% over baseline was observed in five patients: 52%, 58%, 71%, 96% and 99%, respectively. In four of these patients, the creatinine increase to over 50% was detected only once. The mean creatinine increases during the CyA treatment period for these five cases were 9%, 17%, 22%, 10% and 45%, respectively. In the CyA group, serum creatinine concentration at biopsy was 88 μmol/l (range 72–111 μmol/l). None of these patients developed hypertension with CyA.

Kidney biopsies

The detailed scores of renal morphology are given in Table 2 for the patients receiving CyA and in Table 3 for the psoriatic controls. No differences were seen in the incidence of arteriolopathy in the CyA group compared with the controls. The patient who had moderate-to-severe (score = 3) arteriolopathy with CyA (patient 13) had similar lesions before starting therapy (Table 3).

No difference was found between the controls and CyA group as regards the incidence of tubular atrophy or interstitial fibrosis. Minimal tubular atrophy (score = 1) was seen in five out of 16 controls and six out of 14 CyA-treated patients. Slight tubular atrophy (score = 2) and interstitial fibrosis were detected in one out of 16 controls and three out of 14 CyA-treated patients. No case of moderate or severe tubular atrophy or interstitial fibrosis was detected in either group. One patient in the control group had mild nephrocalcinosis. The incidence of obsolescent glomeruli or of glomeruli affected by segmental focal sclerosis was very low and did not differ between the controls and CyA-treated patients. No hypertrophic glomeruli were

TABLE 2. Scoring* of kidney biopsies from psoriatic patients treated with CyA

Patient no.	No. of glomeruli	No. of obsolescent glomeruli	No. of SFGS glomeruli	IF	TA	AP
01	10	2	0	2	2	0
02	5	0	0	0	0	—
03	5	1	0	0	1	0
04	9	0	0	2	2	2
05	17	0	0	1	1	1
06	5	0	0	0	0	0
07	10	0	0	1	0	0
08	13	0	0	1	1	1
09	21	0	0	1	1	0
10	27	0	0	0	0	0
11	13	0	0	1	1	1
12	11	1	0	2	2	0
13	7	0	0	1	1	3
14	5	0	0	0	0	0

SFGS = segmental focal glomerulosclerosis; IF = interstitial fibrosis; TA = tubular atrophy; AP = arteriolopathy.
*0 = absent; 1 = minimal; 2 = slight; 3 = moderate; 4 = severe.

TABLE 3. Scoring* of kidney biopsies from psoriatic patients in control group

Patient no.	Age (years)	Gender	No. of glomeruli	No. of obsolescent glomeruli	No. of SFGS glomeruli	IF	TA	AP
10	39	F	15	0	0	0	0	0
11	21	F	0	—	—	0	0	0
12	43	F	7	1	0	1	1†	0
13	57	M	6	1	0	0	0	3
14	38	M	13	0	0	0	0	2
15	35	F	28	1	0	0	0	0
16	29	M	18	0	0	0	1	0
17	36	M	25	2	0	0	0	0
18	44	M	14	0	0	0	0	0
19	46	F	18	2	0	0	0	0
20	46	M	14	0	0	1	1	0
21	54	M	13	0	0	0	1	1
22	43	M	12	0	1	1	1	1
23	64	F	12	1	0	2	2	1
24	34	M	9	0	0	0	0	0
25	55	F	10	0	0	0	0	1

*0 = absent; 1 = minimal; 2 = slight; 3 = moderate; 4 = severe.
†Mild nephrocalcinosis.
SFGS = segmental focal glomerulosclerosis; IF = interstitial fibrosis; TA = tubular atrophy; AP = arteriolopathy.

observed. In summary, no relevant CyA-associated nephropathy was detected in this series of 14 patients.

DISCUSSION

After the first reports of irreversible structural alterations of the kidney in transplant recipients[6] and in patients treated with CyA for autoimmune diseases,[10] an International Kidney Biopsy Registry of Cyclosporin A (Sandimmun®) in Autoimmune Diseases was started with the aim of collecting all of the relevant material available. The Registry has been successful and, so far, more than 300 biopsies have been collected. All of this material relates to patients treated with CyA for various autoimmune indications. Based on previous reports and analyses of data in the Registry, safety rules were established for the use of CyA in autoimmune indications: doses should not exceed 5 mg/kg/day and should be reduced as soon as the serum creatinine increases by 30–50% over baseline values. All reports of the occurrence of CyA nephropathy in autoimmune diseases were based on initial doses greater than 7 mg/kg/day.[7,11-13] In contrast, in a series of 21 patients receiving CyA 5 mg/kg/day or less for systemic lupus erythematosus, rheumatoid arthritis or related disorders, no morphological changes were detected in the kidney after 17–42 months of treatment.[14] Thus, the therapeutic strategy in psoriasis was designed to ensure safety according to these rules. The results of the first 14 kidney biopsies in CyA-treated psoriatic patients confirmed the validity of the simple guidelines mentioned above. No CyA-induced nephropathy was detected in these 14 patients receiving CyA for 3–37 months at a mean dose of 3·9 mg/kg/day. As observed in a larger series of patients,[4] CyA-associated renal dysfunction, as reflected by the increase in serum creatinine, was reversible after interruption of CyA treatment in all of the patients included in this report (Table I).

Incidentally, control biopsies showed that morphological abnormalities may be found in kidney biopsies of psoriatic patients before treatment with CyA. Such abnormalities usually combine minimal or slight interstitial fibrosis and tubular atrophy and, in one patient, a moderate arteriolopathy was observed. These findings should be taken into consideration when interpreting kidney biopsies from patients treated with CyA for psoriasis as well as for other autoimmune diseases, and emphasizes the need for a standardized evaluation of such biopsies.[15]

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Renal side-effects of cyclosporin A with special reference to autoimmune diseases

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SUMMARY

At therapeutic drug levels, the functional changes which occur are a reduction of glomerular filtration rate and renal plasma flow. At higher doses, morphological changes develop which may result, particularly in severe cases, in acute or chronic renal failure. The threshold for the development of irreversible vascular–interstitial lesions mainly depends on the increment of serum creatinine, age and drug dosage or drug blood level. Based on the experience with cyclosporin A (CyA), the following recommendations have been made for its clinical use, especially in patients with autoimmune diseases. The initial dose should not exceed 5 mg/kg body weight and the dose should be reduced if blood CyA levels are over 250 ng/ml; in addition, a dose reduction is recommended if serum creatinine values exceed 30% of pre-treatment values or if other signs of CyA toxicity, such as hepatotoxicity or hypertension, are found. Strict adherence to these suggestions should allow treatment of patients for prolonged periods without irreversible morphological lesions.

Adverse renal effects with cyclosporin A (CyA) were first noticed by Calne[1] more than 10 years ago, soon after the introduction of the drug into clinical medicine. After initial controversies[2-8] as to the specificity of the morphological lesions, a preliminary classification system[9] was determined. Later, the clinical classifications of acute and chronic were adopted[10-12] which proved, however, to be unsatisfactory. Thus, a morphological basis of classification was resumed.[5,9]

Initially, renal adverse events were termed nephrotoxicity. Although the term is correct, it created a universal impression that the use of CyA always resulted in irreversible renal damage, which is not true. For patients with solid-organ or bone-marrow transplants or life-threatening autoimmune diseases, treatment with CyA and the accompanying risk:benefit ratio has always favoured benefit. With increasing use of CyA in patients with non-life-threatening diseases, it was found that CyA could be used safely without risk of irreversible renal damage. Therefore, the less informative, but less controversial, term—adverse effects or side-effects—was chosen.

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TABLE I. Classification of renal side-effects according to effects of cyclosporin A on the kidney

Functional changes		Structural changes	
Tubular dysfunction	Vascular dysfunction	Tubulopathy (proximal tubule)	Vasculopathy (afferent arteriole)
Common, but reversible	Common, but reversible	Seldom, but reversible	Seldom and irreversible

Mihatsch *et al.*[16]

Still, CyA may cause significant irreversible renal damage if not used in the correct dosages. With more than 10 years of experience in a wide variety of diseases, including autoimmune diseases, CyA therapy is no longer a threat to the kidney with short-term use. However, experience with long-term use in autoimmune disease indications is limited. Therefore, an international biopsy register was established for a standardized re-evaluation of biopsies from patients receiving CyA therapy to more clearly define the potential risks of irreversible structural damage to the kidney.

CLASSIFICATION OF RENAL SIDE-EFFECTS WITH CYCLOSPORIN A

The adverse effects of CyA on the kidney can be classified into two major subgroups: functional and morphological changes. Each of these two subgroups can be further divided into tubular and vascular lesions (Table I). In biopsies from kidney or other transplant recipients as well as from patients with autoimmune diseases, effects unrelated to CyA may also be present, for example, those of rejection, glomerulonephritis, amyloidosis, infection and hypertensive arteriolopathy, and damage due to nephrotoxic drugs, such as analgesics. The morphological lesions unrelated to CyA therapy are indistinguishable from those in patients not receiving CyA.[5,9,10] Any pre-existing renal damage especially involving the vascular tree and the tubulointerstitial space may complicate, or even prevent, identification of CyA-related lesions. In summary, not every lesion seen in the kidneys of CyA-treated patients is drug-related. With the current use of lower drug dosages, it is becoming more difficult to identify CyA-related lesions and, therefore, the biopsy register with a panel of experienced pathologists was established.

The CyA renal side-effects are dose-dependent.[5,13-15] Factors modulating CyA side-effects are age, the presence of additional risk factors such as nephrotoxic drugs and, probably, individual drug sensitivity.[10,16] The aim of the current recommended therapeutic schedules is to restrict the probability of morphological changes to below 10% in CyA-treated patients.

Functional changes encompass side-effects which are not associated with distinct morphological renal lesions and which are usually present even at therapeutic trough levels and dosages. Morphological changes closely related to the CyA dose become superimposed on the functional changes and cause further deterioration of renal function. Although morphological tubular changes are quickly reversible, vascular (–interstitial) lesions may result in irreversible renal damage.

Frequency and time of presentation

In the past, morphological CyA-related lesions were present in more than 50% of biopsies from renal transplant recipients.[14,17,18] The frequency of such lesions in kidney transplant recipients decreased with the use of lower CyA doses to less than 10% of biopsies, and tubular lesions in particular have become a rare finding. In patients with autoimmune diseases, morphological (primarily vascular–interstitial) changes have been found in approximately 20% of these cases (M.J.Mihatsch, unpublished data). However, this register includes biopsies dating back to 1984, when patients were still being treated with dosages of more than 10 mg/kg body weight. In the last 2 years, a decrease in morphological lesions has been encountered as patients are now treated with lower doses.

Functional changes, however subtle, are inevitable with CyA treatment. Morphological tubular lesions may be found whenever creatinine increases to more than 100% above baseline values. Vascular (–interstitial) lesions are rarely seen before the second month of treatment, but may already be present within the first week.[19,20] Interstitial fibrosis is usually found at 6 or more months after starting therapy.[14] However, reliable data concerning the relationship between the time of onset of vascular (–interstitial) lesions, and CyA dose and other risk factors are not yet available.

FUNCTIONAL CHANGES

Clinical findings

Consistent with the dual effects of CyA,[21] clinical findings due to tubular as well as vascular dysfunction are usually present. Tubular dysfunction is reflected by a reduced magnesium and bicarbonate concentration in serum, and an increased potassium and uric acid concentration. Vascular dysfunction, on the other hand, results in increases in serum creatinine and urea, and a decrease in glomerular filtration rate and renal plasma flow. The tubular symptoms are not clinically relevant. Creatinine increases of more than 30%, however, may indicate structural alterations (see below). Hypertension may also be present in up to 20% of patients.

Morphology

Systematic biopsy studies, including electron microscopy, are not available from patients with functional changes alone. On light microscopy, the renal tissue appearances are within normal limits.[5,6,9,14,22,23]

Pathogenesis and aetiology

The mechanisms leading to tubular and vascular dysfunction are the subject of many experimental investigations.[21] Renal findings after a single intravenous administration suggest that CyA causes a pre-glomerular vasoconstriction,[24-26] which may not only explain the rise in serum creatinine, but also the tubular dysfunction which resembles the findings in pre-renal azotaemia.[21] A more generalized vasoconstriction would also explain the eventual rise in blood pressure. The mechanisms responsible at the cellular or even molecular level are as yet unknown.[21]

TUBULAR LESIONS

Clinical findings

These findings do not differ from those of functional toxicity except that serum creatinine may be higher (> 100% of baseline) and there is little evidence of proximal tubular dysfunction.[27]

As tubular toxicity is now rare in kidney biopsies because of the use of low CyA dosages, the morphology is only briefly described. For more details, the reader is referred to earlier reviews.[10]

Morphology

The morphological tubular changes[10] comprise inclusion bodies in tubular epithelial cells corresponding to giant mitochondria, isometric tubular vacuolization and microcalcification (Fig. I). Giant mitochondria predominantly occur in the convoluted parts of the proximal tubule whereas isometric vacuolization is limited to the straight parts of the tubule. The three different lesions may be seen together in one biopsy, in any combination or even in isolation. Tubular lesions are focal and difficult to identify on light microscopy; they are best seen in formalin-fixed tissue using trichrome stains. In typical examples, glomerular, vascular or interstitial lesions are absent. Tubular toxicity may be superimposed, however, on any other lesion.

Although highly characteristic, giant mitochondria, isometric vacuolization and microcalcifications are not specific to CyA therapy, especially when the three different lesions are present in one biopsy. Giant mitochondria may be found in a variety of conditions,[10] and isometric vacuolization is indistinguishable from osmotic nephrosis as seen in patients given mannitol, dextran, sucrose or contrast media.[10]

Aetiology and pathogenesis

An essential aetiological factor in the development of tubular toxicity is a toxic CyA trough level[5,14] exceeding 1000 ng/ml (whole blood measured using polyclonal antibody). In the presence of additional renal damage, in particular ischaemia, co-medication with nephrotoxic drugs and rejection, tubular lesions may be found and usually at non-toxic CyA trough levels.[5,14] Increased individual sensitivity to CyA may also account for tubular lesions at low CyA trough levels in the absence of additional risk factors.[10] Tubular lesions are due to direct tubular damage caused by the parent compound, rather than its metabolites.[28]

VASCULAR–INTERSTITIAL LESIONS

Clinical findings

Vascular-interstitial lesions are the most important and most frequent morphological changes seen at present. The clinical findings are superimposed upon symptoms of functional changes, and appearances are similar in patients with kidney, heart, liver and bone marrow transplants as well as in patients with autoimmune diseases.[13,29-34] Slowly progressive deterioration of renal function and, eventually, hypertension are the most important signs. A further indication of vascular damage may be an increase of factor VIII and anti-thrombin III in the serum,[35-37] and increased platelet deposition is found in kidney grafts with vascular–interstitial lesions.[20] Finally, several cases of haemolytic uraemic syndrome were reported in patients treated with CyA, including those with autoimmune disease.[10,38]

Morphology

Three different morphological lesions have been observed which may occur alone or in combination:[5,14,19,20,39,40] glomerular and/or arteriolar thrombi; CyA-associated arteriolopathy; and interstitial fibrosis (striped form) with tubular atrophy. These lesions have been described in patients independent of the underlying disease being treated with CyA.[22,29,30,33,38,41,42] The morphogenesis is schematically summarized in Fig. 2.

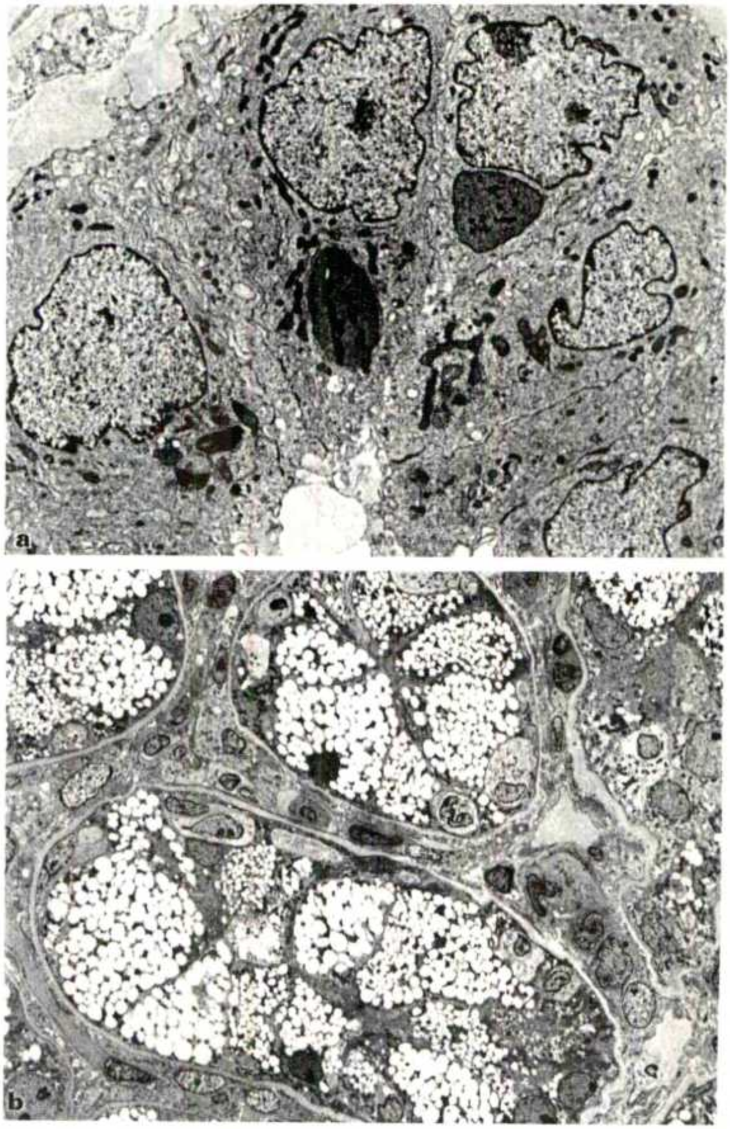

FIGURE 1. Tubular lesions: (a) giant mitochondria and (b) isometric vacuolization. EMs, (a) × 3350; (b) × 850.

Arteriolar or glomerular fibrin or platelet thrombi affect only very few glomeruli or vessels (Fig. 3). Fibrin thrombi have been found in diagnostic kidney-transplant biopsies in approximately 3% of CyA-treated patients.[9,17,39] In later stages, membrane whorls consistent with sequelae of fibrin thrombi may be seen at the vascular pole of the glomerulus. In 25–50% of the patients with thrombi, CyA-associated arteriolopathy is also present.[17,19,20,39]

The hallmark of vascular–interstitial toxicity is CyA-associated arteriolopathy and interstitial fibrosis (striped form) with tubular atrophy.[5,14,43,44] The vascular lesions predominate in the

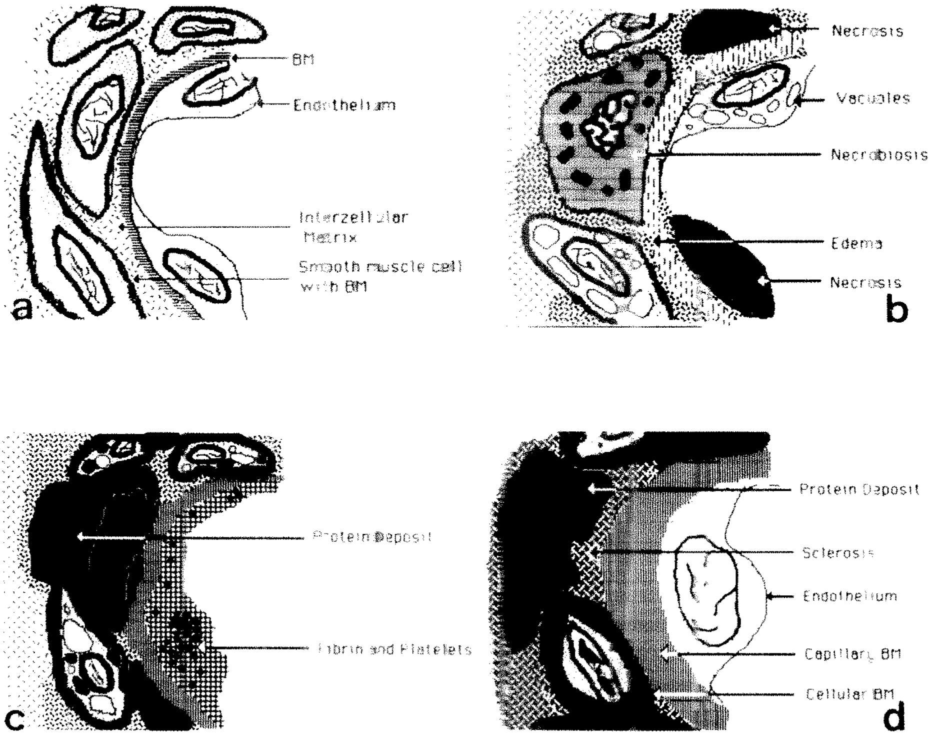

FIGURE 2. Morphogenesis of CyA-associated arteriolopathy: (a) part of a normal arteriole; (b) early lesion consisting of vacuolization, including bodies and endothelial cell necroses; (c) fibrin and platelet thrombi on the denuded basement membrane and protein insudation into the vessel wall; (d) after regeneration of the endothelial cell, protein deposits persist within the vessel wall.

peripheral vascular tree; arterioles (afferent vessels) and arteries with up to two layers of smooth muscle cells are almost exclusively affected. The lesion may extend to the vascular pole of the glomerulus, involving some glomerular segments, and into arteries where they branch into arterioles. Proliferative arteriopathy of interlobular and arcuate arteries[45] is not a feature of CyA toxicity.[39]

Intimal fibrosis in arteries, however, is common in cases of severe and long-standing CyA-associated arteriolopathy. Cyclosporin A-associated arteriolopathy occurs in two forms which may be co-existent.[5,14] In most cases, nodular protein deposits permeate the arteriolar wall, thus narrowing or even occluding the vascular lumen. These deposits are often arranged in a pattern resembling a pearl necklace or clover leaf (Fig. 4). Electron microscopy reveals that these protein deposits have replaced necrotic smooth muscle cells (Fig. 5). They consist of IgM and/or complement (C3/C1q) and, occasionally, fibrin (Fig. 6). In cases of mild CyA-associated arteriolopathy, the distinction from arteriolar hyalinosis becomes very difficult. The second form is characterized by a mucoid thickening of the intima, resulting in narrowing of the vascular lumen (Fig. 7); this is very rarely seen in patients with autoimmune diseases.

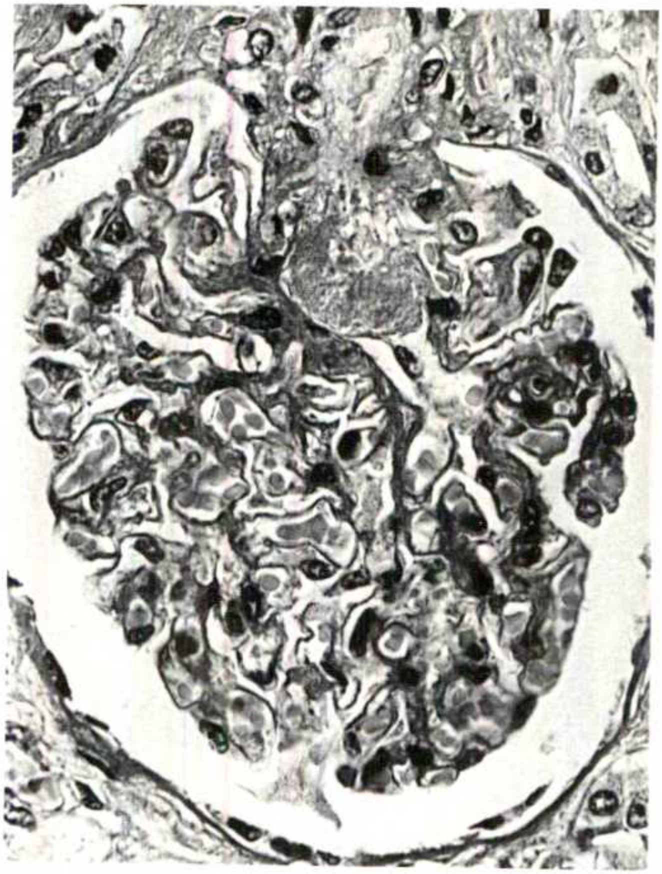

FIGURE 3. Fibrin thrombus on the vascular pole of the glomerulus. EM, AFOG stain, (× 500).

The end result of the vascular lesion may be complete occlusion of the arterioles (Fig. 8), which eventually vanish within the interstitial fibrosis. In both types of CyA arteriolopathy, but possibly also in their absence, minor changes in endothelial and smooth muscle cells may be present, including vacuolization, single-cell necrosis and inclusion bodies corresponding to lysosomes.

Cyclosporin A-associated arteriolopathy is accompanied or followed by interstitial fibrosis (Fig. 9). Irregular foci or stripes of interstitial fibrosis with atrophic tubules are observed in the renal cortex,[5,8,14,15] while tubules in other areas appear essentially normal. A sparse mononuclear cell infiltrate is often seen in the fibrotic areas not only in patients with kidney transplants, but also in those with autoimmune diseases who have primarily extrarenal manifestations. Intimal fibrosis may be seen in arteries, and glomerular changes are usually not conspicuous, but become more frequent and prominent (obsolescent glomeruli, segmental focal glomerulosclerosis) in cases of advanced interstitial fibrosis and tubular atrophy. Typical CyA-associated arteriolopathy or, more often, completely scarred arterioles or atypical arteriolar lesions may also be found.

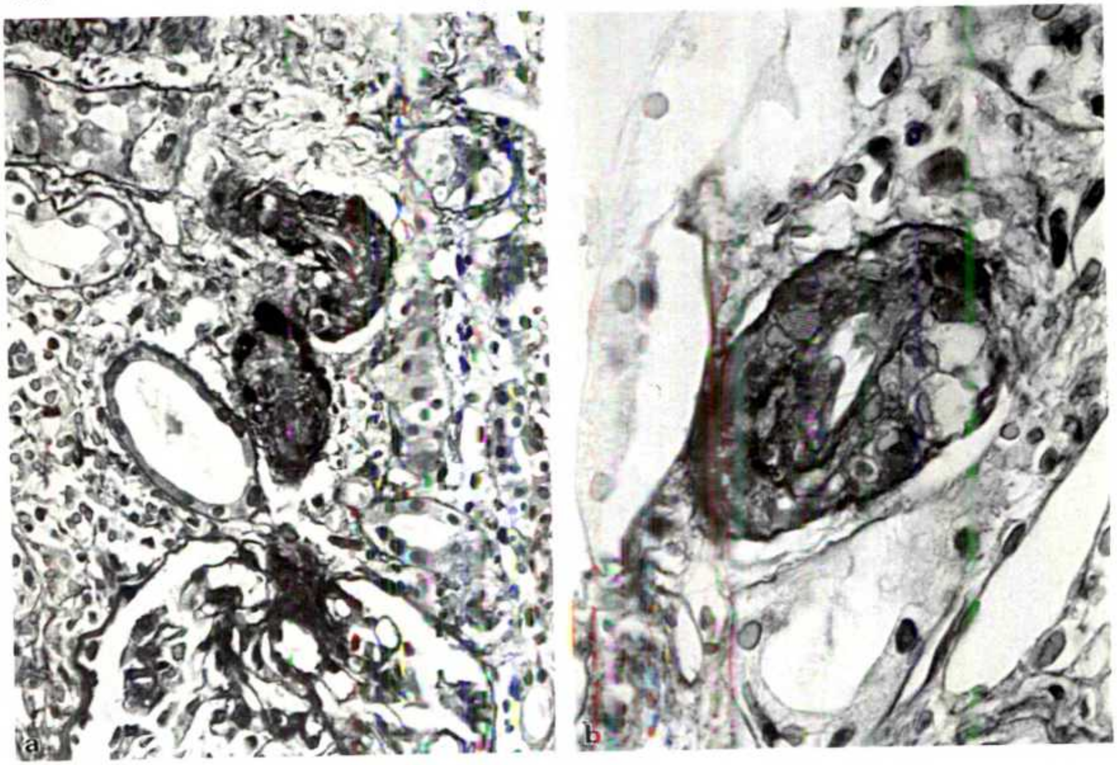

FIGURE 4. Glomerulus with afferent arteriole permeated by nodular protein deposits and narrowing of the vascular lumen. EM, AFOG stain, (a) × 347; (b) × 801.

Differential diagnosis

The morphological lesions seen in vascular–interstitial toxicity are non-specific; CyA-associated arteriolopathy is very similar to that seen in cases of thrombotic microangiopathy without CyA treatment.[46] Cyclosporin A-associated arteriolopathy and hypertensive or diabetic arteriolar hyalinosis is usually easily differentiated because of the presence of focal nodular deposits within an intact smooth muscle cell layer in hyalinosis,[47] in contrast to nodular deposits replacing necrotic smooth muscle cells in CyA-associated arteriolopathy. In advanced cases of hypertensive or diabetic arteriolopathy, or in late stages or mild forms of CyA-associated arteriolopathy, however, differentiation using light microscopy may be either difficult or impossible.

Pathogenesis and aetiology

The pathogenesis of vascular–interstitial lesions is still poorly understood. The morphology in human biopsies and the experimental findings suggest primary endothelial and smooth muscle cell damage of the vasoconstricted afferent vessels.[40] This process may result in a subclinical and recurrent thrombotic microangiopathy,[10,48] an assumption further supported by the increased deposition of platelets in patients with kidney transplants, and CyA-associated arteriolopathy with an increased urine excretion of thromboxane, indicating platelet degradation.[20,49] Some patients receiving CyA develop the typical clinical picture of haemolytic uraemic

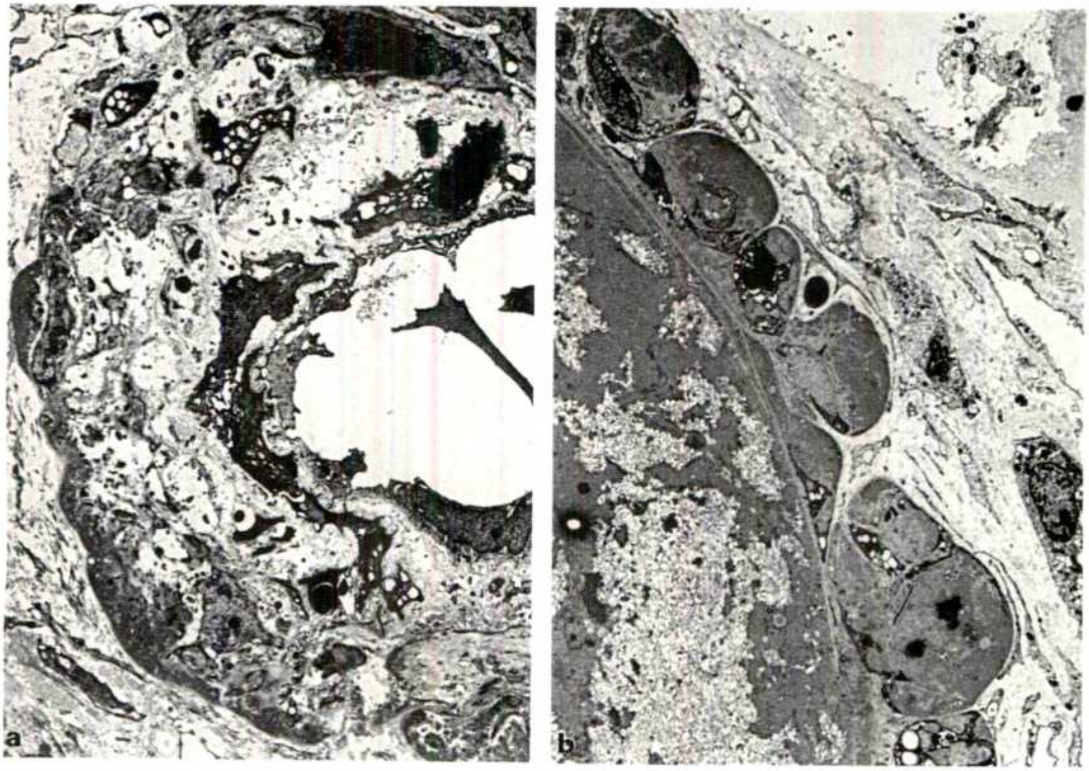

FIGURE 5. Early and late stages of CyA-associated arteriolopathy: (a) extensive necrosis of the smooth muscle cells within the vessel wall and early protein deposits; (b) advanced stage with nodular protein deposits replacing smooth muscle cells, and occlusion of the vascular lumen by fibrin and protein thrombus. EM. × 2350.

syndrome.[10,36] Interstitial fibrosis with tubular atrophy is the result of chronic ischaemic damage of the tubulointerstitial space due to CyA-associated arteriolopathy.[5,14,50]

RISK-FACTOR ANALYSIS AND RECOMMENDATIONS

The major risk factors observed in the different groups of patients receiving CyA are summarized in Table 2.[34,51-53] The most important factors are age, percentage increase of serum creatinine over baseline, dosage and/or blood levels of CyA, hypertension, pre-existing or additional renal damage such as ischaemic renal failure, kidney irradiation and co-medication of nephrotoxic drugs (for example, antibiotics and non-steroidal anti-inflammatory drugs). As patients with psoriasis usually have no pre-existing renal pathology, the most important factor is the maximum increment of serum creatinine (30% over baseline). In patients treated for autoimmune diseases, there is a correlation between the incidence of vascular–interstitial lesions and the extent of serum creatinine increase: the incidence of vascular–interstitial renal lesions was 6·5%, 11% and 40% in patients with creatinine increases of 30%, 30–50% and 50–75%, respectively.

As regards age, children appear to be less prone to developing vascular–interstitial lesions;

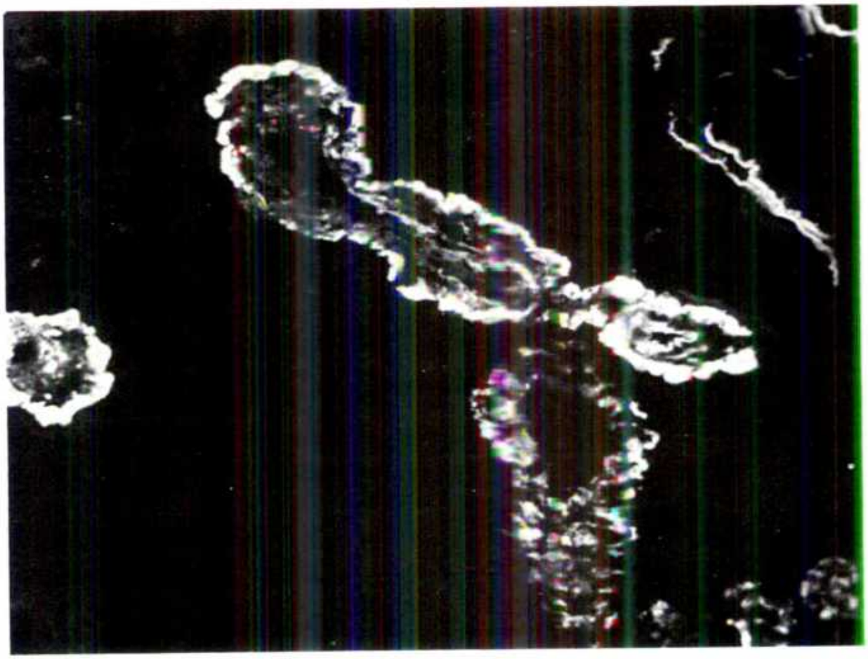

FIGURE 6. Immunofluorescent findings of circular nodular complement deposits in arterioles. EM. × 500.

this may be due to either a lower drug sensitivity or a decreased bioavailability due to higher metabolic rates in childhood. Thus, serum creatinine increases are lower in children than in adults for a given dose. However, the risk of developing vascular–interstitial lesions in patients with comparable reductions of renal function is identical for children and adults.[34]

The size of the dose is the third most important risk factor in CyA therapy. At a daily dose of less than 5 mg/kg body weight, no significant vascular–interstitial lesions were found. At higher doses of CyA, 5–7·4 mg/kg and 7·5–19·9 mg/kg, the prevalence of vascular-interstitial lesions increased to 13% and 29%, respectively.[16]

Current recommendations for the treatment of patients with autoimmune diseases, including psoriasis, should take into consideration the above risk-factor analysis. Treatment should begin with doses of 5 mg/kg or less with careful monitoring of serum creatinine concentrations. If serum creatinine increases to more than 30% over baseline, dosage should be adjusted. If hypertension develops, pharmacological control should be instituted or CyA therapy must be stopped.

CONCLUSIONS

Cyclosporin A may produce a new form of drug toxicity which is dose-dependent. At low dosages or blood levels, functional changes prevail, consisting of reductions in glomerular filtration rate and renal plasma flow. Superimposed over these functional changes are morphological alterations of the proximal tubule and, more importantly, of the vascular–interstitial space which may develop at high CyA dosage and/or drug levels. The likelihood of developing vascular–interstitial lesions resulting in progressive renal failure depends on dose, concomitant

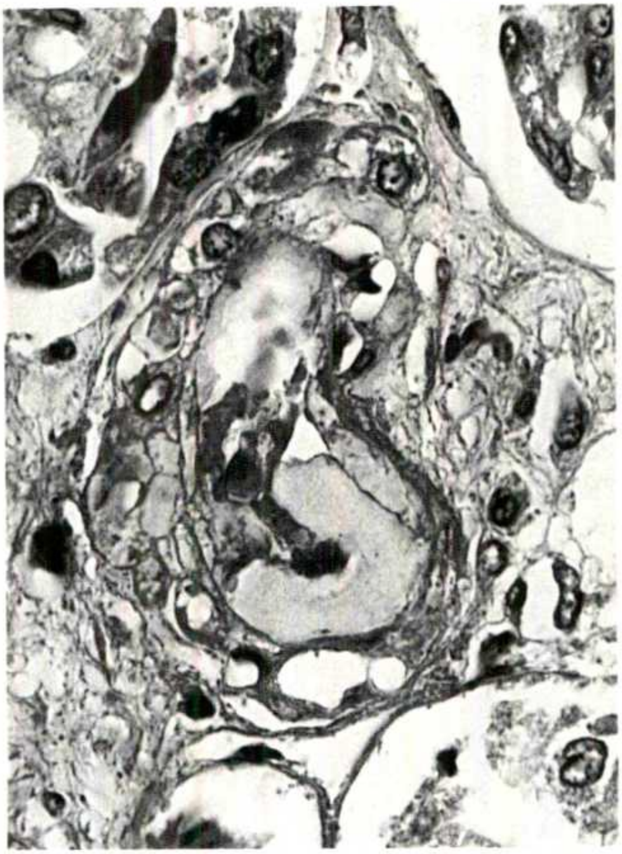

FIGURE 7. Rare form of CyA-associated arteriolopathy with mucoid narrowing of the vascular lumen. Note extensive necrosis of the vascular smooth muscle cells. EM, AFOG stain, × 900.

risk factors and individual sensitivity to the drug. If these recommendations are strictly followed, CyA therapy does not present a major risk to the kidney.

SPECIAL NOTE

International Kidney Biopsy Registry of Cyclosporin A (Sandimmun®) in Autoimmune Diseases

In order to obtain a standardized evaluation of kidney biopsies in patients treated with CyA for autoimmune diseases, an Advisory Board has been organized by nephropathologists who already have wide experience in this field. Various geographical regions have been defined and assigned to the specialized pathologist who will review all kidney biopsies in that region. At the constituting session, the members of the Advisory Board of pathologists established a catalogue of morphological findings which may occur after CyA therapy. All physicians wishing to contribute to the Kidney Biopsy Registry will be asked to provide unstained sections or, if possible, tissue blocks of appropriately fixed biopsy material to the regional review pathologist or to any pathologist on the Advisory Board. The tissue blocks will be returned within 1 month

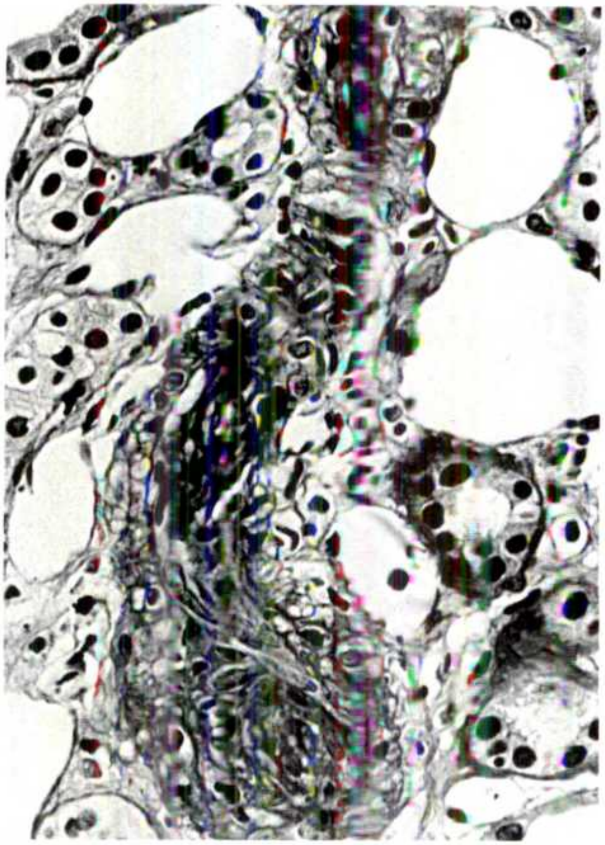

FIGURE 8. Advanced stage of CyA-associated arteriolopathy with complete occlusion of the vascular lumen. EM, PAS stain, × 450.

together with the interpretation of the reviewing pathologist. A few representative sections will be permanently retained for meetings of the Advisory Board.

Review pathologists

Children worldwide
  Dr R.Habib, Hôpital des Enfants Malades, Paris, France
France (adults)
  Dr L.H.Noel, Hôpital Necker, Paris, France
Germany
  Dr U.Helmchen, Universitätsklinik Eppendorf, Hamburg, FRG
Scandinavia and Great Britain
  Dr S.O.Bohman, Karolinska Institutet, Huddinge, Sweden
U.S.A. (East Coast)
  Dr T.Antonovych, Armed Forces Institute of Pathology, Washington, DC, U.S.A.
U.S.A. (West Coast)
  Dr R.Sibley, Stanford University, Palo Alto, California, U.S.A.
Other countries

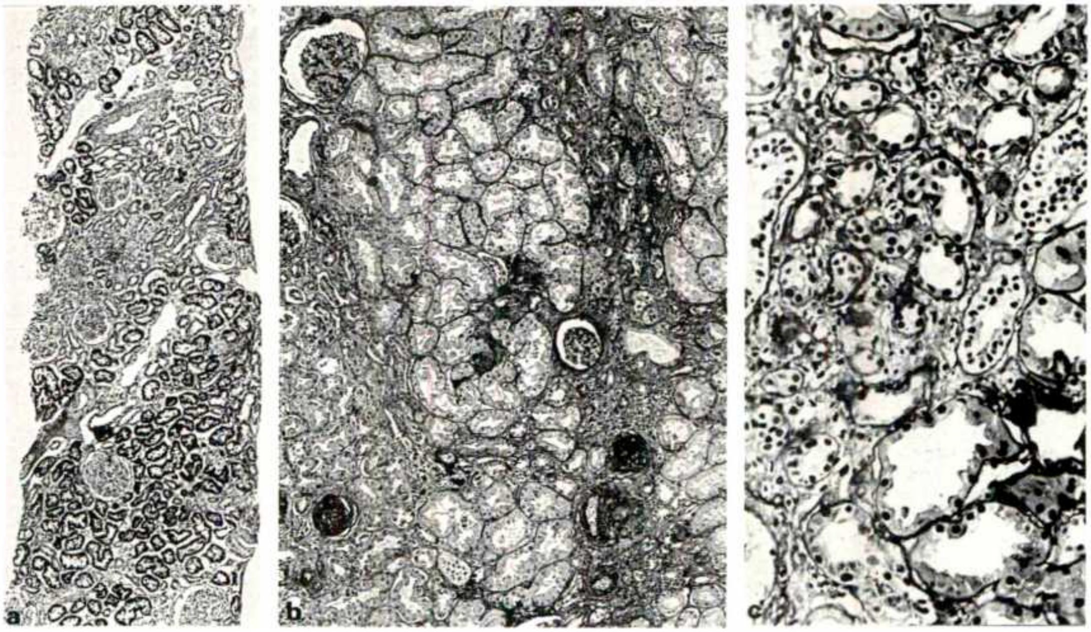

FIGURE 9. Striped form of interstitial fibrosis with tubular atrophy seen at three magnifications: (a) renal biopsy with severe fibrosis and tubular atrophy (between black lines); (b) higher magnification shows adjacent atrophic tubules surrounded by fibrosis and well-preserved renal tissue; (c) atrophic tubules exhibit mostly thickened basement membranes. EMs, PAS stain, (a) × 30; (b) × 60; (c) × 163.

TABLE 2. Risk factors of vascular–interstitial lesions in CyA-treated patients: a summary of various studies

	Kidney transplant	Bone-marrow transplant	Heart transplant	Autoimmune disease
Age	—	—	—	+
CyA ARF	+	+	+	+
CyA TL (long-term)	+	+	+	+
Nephrotoxic drugs (ARF)	+	+	—	—
Ischaemic ARF	+	—	—	—
Hypertension	—	+	—	+
Pre-existing renal damage (> 200 μmol/l)	—	—	—	+
Irradiation	—	+	—	—

ARF = acute renal failure or percentage increase in serum creatinine; TL = CyA trough level.

Dr M.J.Mihatsch, University of Basle, Basle, Switzerland (Organizer of the Advisory Board)

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Side-effects of psoralen photochemotherapy (PUVA)

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SUMMARY

Although psoralen photochemotherapy (PUVA) is one of the most effective forms of therapy for psoriasis, the risk of potential long-term side-effects is, as yet, not clearly determined. Chronic degenerative and pigmentary skin changes similar to those of chronic solar exposure occur after long-term PUVA treatment; PUVA also causes non-melanoma skin cancers in man, although there is, as yet, no consensus as to what cumulative phototoxic PUVA dose is carcinogenic. Long-term multicentre studies from the U.S.A. indicate a definite risk of squamous cell carcinoma for long-term PUVA-treated patients, whereas European studies reveal no overall increase in skin cancers in similar patients except for those exposed to other carcinogens. Assignment to PUVA should be based on the risk:benefit ratio for the individual patient. Careful patient selection is therefore mandatory and should be limited to those who can be monitored and controlled by informed, competent and conscientious physicians.

Psoralen photochemotherapy (PUVA)[1] is one of the most effective forms of therapy for psoriasis.[2] It induces a complete clinical remission within a reasonably short period of time and, as maintenance therapy, can produce a remission for an extended period. In combination with retinoids,[3] the clinical efficacy of PUVA is greatly improved and is likely to minimize the potential long-term side-effects.

There is now considerable clinical experience regarding the short-term safety of PUVA, but the risk of potential long-term side-effects is, as yet, not clearly determined.

ACUTE SIDE-EFFECTS AND TOXICITY OF PUVA

Side-effects (Table 1) are related to overdosing with long-wave ultraviolet (UVA) radiation or the patient's inability to tolerate the psoralens used in the treatment. The most frequent side-effect due to overexposure is erythema.[4] Pruritus is also UVA dose-related, and a few patients experience painful burning sensations in the exposed skin towards the end-phase of treatment.

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TABLE 1. Immediate side-effects of PUVA ($n = 3175$)

Side-effect	Frequency (%)	Discontinuation of PUVA (%)	Interruption (clearing phase) (%)	Interruption (maintenance phase) (%)
Erythema	32·4	1·7	5·7	0·3
Pruritus	25·6	1·4	2·9	0·4
Nausea	13·5	0·6	1·5	0·3
Headache	2·0	0·1	0·1	0·1
Koebner reaction	2·0	0·2	0·5	0·1

Henseler *et al.*[9]

Overdose phenomena usually arise in areas not normally exposed to natural sunlight. Careful observation of the criteria for dosimetry and the guidelines for treatment[5] will reduce these adverse events.

Transient nausea is relatively common with 8-MOP (8-methoxypsoralen; methoxsalen) used as a photosensitizer,[4] but occurs only rarely with 5-MOP (5-methoxypsoralen).[6] Only on rare occasions is the nausea so severe as to necessitate discontinuation of treatment. Other short-term side-effects include acne-like eruptions, polymorphous light eruption-like rashes, subungual haemorrhages due to phototoxic reactions and transient hypertrichosis of the face.[7] These are all reversible and disappear on discontinuation of treatment.

Laboratory data

As psoralens in excessive doses are hepatotoxic in laboratory animals, there has been concern in the past as to liver damage in humans. Laboratory examinations performed over many years have not revealed evidence of impaired hepatic function and, although a slight increase in blood urea nitrogen (BUN) and creatinine has been documented in one series, there is no evidence to suggest impairment of renal function in large-scale studies.[8,9] Analysis of the laboratory data from several studies has not revealed significant abnormal findings in those patients who have received PUVA over a prolonged period of time (Table 2). One study reported an increased incidence of antinuclear antibodies in PUVA-treated patients,[10] but other studies have shown no such relationships.[11-13]

Potential long-term risks of PUVA

On the basis of *in vitro* experiments and animal studies, psoralens and UVA are known to have mutagenic and carcinogenic effects.[14-17] It is to be expected that these effects are of relevance to man. Potential long-term side-effects of PUVA therapy include chronic actinic skin damage, cutaneous tumours, ocular damage and immunological alterations. It is assumed that these adverse effects depend on the total cumulative phototoxic dose received over a prolonged period of time and are probably only apparent after an extended period of latency.[2,7]

Chronic actinic skin damage

Chronic PUVA treatment may produce changes in the skin that resemble premature ageing induced by sunlight. Both dermal and epidermal changes have been reported, but studies of chronically exposed skin have revealed contradictory results. A phenomenon described as focal epidermal dystrophy[18] was reported in one study to affect 50% of patients treated for up to 1

TABLE 2. Laboratory test results from the U.S.A. 16-centre PUVA study*

Test	Positive change	No change	Negative change	*P* value (sign test)	Mean change
Haematocrit	422	133	363	< 0·05	+ 0·27
WBC	396	31	494	< 0·01	− 0·16
Bilirubin	359	138	414	< 0·1	− 0·005
Alkaline phosphatase	392	59	463	< 0·05	− 4·83
SGOT	411	70	463	> 0·2	− 1·06
LDH	350	32	445	< 0·001	− 5·15
BUN	400	86	269	< 0·001	+ 0·79
Creatinine	370	193	302	< 0·01	+ 0·01
Uric acid	367	47	498	< 0·001	− 0·16

*Melski *et al.*[8]

WBC = white blood cell count; SGOT = serum glutamic-oxaloacetic transaminase; LDH = lactate dehydrogenase; BUN = blood urea nitrogen.

year, but these changes have not been consistently found in other studies[12,13] and, when observed, were seen only in patients who had received an extremely high radiation load.[19] Focal epidermal dystrophy consists of abnormally large hyperchromatic nuclei in epidermal cells either singly or in clusters, focal disorientation of keratinocytes and atypical cells with lobulated or multiple nuclei. It is not known whether such foci of epidermal derangement represent precursor lesions for actinic keratoses or epithelial tumours. In one study,[20] it is of interest that epidermal dystrophy did not increase with increasing exposure. Reversal of these changes has been observed after cessation of PUVA therapy.[19]

Dermal changes are the most consistent finding in chronically PUVA-treated skin.[12,21] These include eosinophilic homogenization of the dermo-epidermal junction, reduction and fragmentation of elastic fibres and an accumulation of an amorphous, eosinophilic material around blood vessels. A certain dose-dependency of these changes has been documented[12] and it is reasonable to assume that dermal changes induced by PUVA add to the injury induced by natural sunlight.

Changes of the epidermal pigmentary system induced by chronic PUVA include a clustering of melanocytes, an increase in melanocyte size and heavy melanization of keratinocytes.[22] The increase in the number of melanocytes and dermal melanophages noted in chronically PUVA-treated skin directly correlate with the total UVA dose used.[12] No changes have so far been observed in melanocyte structure that suggest early premalignant degeneration.

Long-term PUVA patients may develop widespread lentigines which are different from ordinary solar lentigines. An increased proportion of large melanocytes with aberrant melanosome changes has been reported as well as an increase in the average size of melanosomes and the occurrence of giant melanosomes.[23] It is not known whether these melanosome changes represent a reversible phenomenon or evidence of somatic mutation.

PUVA-induced mottling is a pigmentary phenomenon observed in a minority of patients and is mainly, but not invariably, restricted to areas of overdosage.[12] It consists of hyper- and hypopigmentation combined with atrophy. The accumulation of lipids in the hyperplastic melanocytes of PUVA-treated skin may be a result of overstimulation leading to melanocyte destruction,[24] resulting in mottling in the hypo- and depigmented areas.

TABLE 3. PUVA and non-melanoma skin cancers*

Authors (Ref)	Year	Observation period (weeks)	n	Tumours (%)	BCC/SCC
Stern (29)	1979	2	1373	2·2	1/1·3
Hönigsmann (32)	1980	5	418	1·2	1/3
Roenigk (37)	1981	4	631	1·6	1/3
Lassus (35)	1981	2	525	0·2	1/0
Ros (36)	1983	4	250	0	0
Stern (30)	1984	6	1286	8·1	1/1·1
Eskelinen (39)	1985	6	1047	0·4	1/1
Tanew (40)	1986	8	297	3·3	1/2
Henseler (41)	1987	8	1643	2·1	1/1
Stern (31)	1988	10	1380	11·7	1/1·8
Forman (38)	1989	10	551	3·8	1·5/1

*Selected studies.
BCC/SCC = basal cell carcinoma/squamous cell carcinoma.

Carcinogenesis

The photochemical reaction in PUVA involves the interaction of psoralens with DNA which has been shown to produce mutations in bacterial systems[14,15] and in mammalian cells.[25] PUVA induces an increased number of sister chromatid exchanges *in vitro*,[26] but such changes have not been observed *in vivo* during therapy.[27] 8-methoxypsoralen plus UVA is carcinogenic in animals and it has been shown that, with topical or intraperitoneal application of 8-MOP, the rate of skin tumours is higher than after oral administration.[16,17,28] The induction or promotion of skin cancers in patients treated over prolonged periods of time with PUVA therefore represents an area of concern.

A 16-centre cooperative study in the U.S.A. reported an increased risk of non-melanoma skin cancers in patients treated with PUVA.[29] This increased risk was particularly evident in patients with a previous history of ionizing radiation or previous cutaneous carcinoma. The risk was apparent in the second year after the initiation of PUVA and has been sustained dose-dependently after 5 and 10 years.[30,31] In the initial study, the incidence of squamous cell carcinomas was significantly higher than that expected in the general population and particularly high in those areas of the skin not usually exposed to the sun. On the other hand, the incidence of basal carcinomas did not differ initially from that expected in the general population.[29] Indeed, no overall increase in skin cancers has been observed in several large-scale European and other U.S.A. studies during the first 5 years of follow-up (Table 3). The development of skin cancers in PUVA-treated patients appeared to be related to particular risk factors, such as previous ionizing radiation and arsenic intake.[32-37] The initial question raised, therefore, was whether PUVA in humans is oncogenic by itself or only a promoter in those patients who carry a genetic predisposition or who have been exposed to other risk factors, such as excessive solar radiation, arsenic and ionizing radiation. The short latency period between PUVA treatment and carcinoma development in the first 16-centre U.S.A. study[29] was considered an argument against PUVA as the initiator of carcinogenesis and was interpreted as a promoter effect.[2] However, long-term evaluation of the same patient material[30,31] has now shown a definitely increased risk of squamous cell carcinoma in long-term PUVA-treated patients, a conclusion supported by another, independent, multicentre trial from the U.S.A.[38] Of 1380 patients observed for 10 years after first exposure to PUVA, 162 were found to have a total of 391 squamous cell carcinomas and 218 basal cell carcinomas, demonstrating a strong association between cumulative exposures to PUVA and increased carcinogenesis.[31] For tumours occurring at least 58 months after the initial treatment, an elevenfold increase in risk was found for patients who had received more than 260 treatments compared with individuals who had received 160 or fewer treatments during the same time interval.[31] (Six per cent of the cohort, who had received more than 260 treatments by the fifth year of follow-up, averaged more than 7000 J/cm^2 by the end of the study period!)

In contrast, large-scale European studies have not found an increased risk for squamous cell carcinomas in long-term PUVA-treated patients unless they belong to the risk groups mentioned above.[39-41] An exceptionally high risk for developing skin cancers has been reported for two patients who had received both methotrexate and PUVA.[42] In the European PUVA study, for instance, which involved an 8-year follow-up of 1643 patients and an average observation period of 96 months, only 36 patients were observed to have a total of 71 tumours (40 squamous cell carcinomas, 23 basal cell carcinomas, one keratoacanthoma and one melanoma).[41] Nearly all of the patients with tumours had been exposed to various carcinogens before the initiation of PUVA and no tumours were detected in patients without such previous treatment, although 10% of the patients had received more than 3000 J/cm^2 as a total cumulative UVA dose.

This apparent discrepancy between the U.S.A. and European experience is enigmatic, but may be explained by several variables, such as patient selection (ethnic background, previous exposure to potential carcinogens, solar exposure), treatment schedules and light sources. The smaller percentage of skin Type I patients in the European series[40,41] and the more aggressive treatment schedule (fewer, but higher, phototoxic PUVA doses), which is less carcinogenic in the murine model,[43,44] may account for the lower incidence of squamous cell carcinomas in these series. There is consensus that tumours developing in long-term PUVA-treated patients are biologically not aggressive and, so far, metastasis has occurred in only two patients.[31] PUVA-induced tumours are small, slow-growing and removable by simple excision.

The main concern related to oncogenic risk of photochemotherapy is malignant melanoma. Although morphological data in some studies have apparently indicated irreversible structural changes in melanocytes, malignant melanomas have been observed in only a few PUVA-treated patients[41,45,46] and, so far, there is no evidence to support an increased frequency of melanoma in patients exposed to PUVA for prolonged periods of time.[46]

Immunological effects

PUVA has been shown to alter immune responses to a variable and minimal degree which has not been clinically relevant. PUVA causes dose-dependent changes in the function of lymphocytes[47-49] in both experimental animals and man, possibly as a result of impaired interleukin-2 (IL-2) production,[50] and diminishes the induction of allergic contact dermatitis and delayed hypersensitivity.[51-55] Full-thickness graft survival has been shown to be prolonged in animals after exposure of the donor and recipient sites to PUVA.[56] Langerhans cell function may be impaired and a reduced number of Langerhans cells has been reported in PUVA-treated experimental animals, human volunteers and psoriatics, using either adenosinetriphosphatase (ATPase) or major histocompatibility complex (MHC) Class II molecules as identification markers.[57-61] A reduction of the overall epidermal allostimulatory capacity has also been reported.[62]

PUVA may suppress immunological surveillance not only locally in the skin, but also systemically, although such changes are reversible and their clinical significance not yet known. Indeed, this 'immunosuppression' may explain the therapeutic success of PUVA in diseases other than psoriasis.[7] However, there is speculation that this impaired immunological function may contribute to the development of skin carcinomas in long-term PUVA therapy.[7,51]

There is also controversy concerning the possible effects of PUVA on the function of neutrophils in antibody production and immune complexes, and a potential induction of autoimmune disease.[7] Unmasking of subclinical systemic lupus erythaematosus and bullous pemphigoid by PUVA has been reported,[62-66] but several large-scale multicentre studies have presented strong evidence against a possible relationship between PUVA and the induction of autoimmune disease.[11-13] Other than an increased incidence of recurrent herpes simplex, there is no evidence of an increased risk of bacterial, viral or fungal infections in patients exposed to photochemotherapy for prolonged periods of time.

Ophthalmological effects

High-intensity UVA can lead to cataract formation, and animal experiments have indicated a potential ocular toxicity in association with PUVA. Psoralens can be detected in lens tissue, and photoproducts have been demonstrated in lens protein in rats on exposure to UVA which may accumulate and remain permanently within the lens.[67,68] Cataracts have been produced in experimental animals after high doses of 8-MOP and UVA, but not with doses comparable to those used in photochemotherapy in man.[68-71]

So far, there is no convincing evidence that psoralen-induced cataracts may occur in patients subjected to long-term photochemotherapy.[7] In the U.S.A. photochemotherapy follow-up study, there was no evidence of an accelerated disturbance of visual acuity in 1235 patients examined at least 1 year after starting therapy.[8] It is noteworthy that in a follow-up of 13 patients who had been treated for vitiligo with 8-MOP and natural sunlight, no eye changes were seen from between 2 and 12 years after initiation of therapy, despite the fact that eye protection had not been recommended.[72] To avoid eye toxicity, it is preferable that appropriate UVA-opaque spectacles be worn during the entire period of increased photosensitivity after psoralen ingestion.[73] At present, the indications are that, if eye protection is provided, PUVA will not induce ocular toxicity in humans.

CONCLUSIONS

Photochemotherapy is a highly effective treatment for severe psoriasis. While there is a comprehensive clinical experience documenting PUVA short-term safety when used according to standardized methods, the risk of potential long-term side-effects has not yet been ascertained. In view of these potential long-term hazards, the assignment of patients to photochemotherapy should be based on consideration of the risks and benefits for each individual patient. Careful patient selection, therefore, appears to be mandatory. According to the guidelines issued by the Task Force on Photobiology of the American Academy of Dermatology,[74] patients should have severe psoriasis with a ≥ 30% body involvement. The condition should be disabling physically, emotionally and economically, and so severe that it cannot be adequately managed by conventional antipsoriatic treatments. It is also preferable to limit PUVA to older patients.[74] However, these guidelines are not strictly adhered to as far less than a 30% body involvement is often disabling, and psoriasis can be particularly devastating in younger patients. Concern should primarily be centred on long-term treatment and the avoidance of excessive total cumulative PUVA doses by the use of combination treatments[2,3] or by cycling patients through different treatment modalities.

Severe impairment of hepatic and renal function is usually considered a contraindication to PUVA because metabolism and excretion of psoralens may then be inadequate. Patients with chronic actinic damage and a history of skin cancers may bear a higher risk for the development of new cancers, and previous arsenic intake and ionizing radiation also increase the risk of non-melanoma skin cancers. Immunosuppressed patients should probably not receive PUVA as the treatment may add to the already existing immunosuppression.

Severe widespread psoriasis is a devastating disease which may result in disruption of professional, social and private life. After 16 years of experience with PUVA for the treatment of psoriasis, however, it has become evident that this therapy offers patients the chance to resume normal lives although, in view of the potential risks, they should be carefully selected, and the treatment limited to those who can be monitored and controlled by informed, competent and conscientious physicians.

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Methotrexate side-effects

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SUMMARY

Methotrexate is by far the most widely used cytotoxic drug in psoriasis. Treatment requires normal kidney, liver and bone-marrow function, and pregnancy and alcohol abuse are absolute contraindications. Serious toxic reactions are recognized, but can be avoided if the drug is used correctly. The most important side-effects are haematopoietic and hepatotoxic. It is well established that long-term methotrexate can induce liver damage which, in a number of patients, may lead to fibrosis or cirrhosis. Recent studies have, however, documented that the methotrexate-induced liver cirrhosis is not aggressive. Interaction can occur with a number of drugs; serious problems in particular may arise with concomitant use of sulphonamides and salicylates. The recommended guidelines for methotrexate use in psoriasis should be followed and patients given clear instructions.

Cytotoxic drugs have been used against psoriasis for more than 34 years. Among these, methotrexate (MTX), introduced in 1958,[1] is by far the most widely used. The arrival of psoralen and long-wave ultraviolet light (PUVA), retinoids and now cyclosporin A (CyA) has undoubtedly reduced the use of MTX. The drug, however, is still very much in use because of its efficacy and a high degree of patient compliance. Among the various treatment modalities for severe psoriasis and psoriatic arthritis, the longest experience is with MTX.

The immune system is a possible target for the antipsoriatic effects of MTX. Other possible working mechanisms are interference with epidermal cell kinetics, presumably by a temporary reduction in DNA synthesis, a decrease in neutrophil and monocyte chemotaxis, and inhibition of the C5a-induced skin response and LTB_4-induced intraepidermal penetration of granulocytes.[2] Methotrexate may be given orally once weekly in a single or divided dose, or in a once-weekly parental dose. Treatment depends on normal kidney, liver and bone marrow function, and pregnancy and alcohol abuse are absolute contraindications. An active peptic ulcer or severe infection should also not be present. A summary of the exclusion criteria for MTX use is seen in Table 1.

Serious acute toxic reactions have been recognized, but can be avoided if the drug is correctly used. Methotrexate may lead to long-term side-effects, mainly in the form of liver toxicity

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TABLE 1. Exclusion criteria for methotrexate in psoriasis

- Pregnancy
- Renal impairment
- Recent hepatitis
- Pre-methotrexate cirrhosis of the liver
- Excessive alcohol intake
- Patient unreliability
- Unwillingness to adopt control measures
- Unwillingness to use contraception
- Active peptic ulcer
- Severe infections
- Pronounced anaemia
- Leukopenia
- Thrombocytopenia

(Table 2). Except for complaints of gastrointestinal origin, there appear to be no differences in incidence and severity of side-effects among the dosage schedules currently used in psoriasis.

SUBJECTIVE SIDE-EFFECTS

These effects can be seen from the first week as well as later during treatment. Typically, they arise on the same day of or within the first few days following MTX administration. Nausea and abdominal discomfort are common while fatigue, headache and vomiting are less common, and depression as well as anorexia are rare. In cases of gastrointestinal problems, changing from oral to parenteral administration may be helpful.

HAEMATOPOIETIC SIDE-EFFECTS

Haematopoietic suppression with the MTX dosages used in psoriasis is not frequently encountered. Leukopenia occurs more frequently than thrombocytopenia. Anaemia rarely arises and

TABLE 2. Side-effects of methotrexate in psoriasis

Common and mild	Uncommon	Rare
Nausea	Fatigue	Stomatitis
Abdominal discomfort	Headache	Burning sensation
Leukopenia	Vomiting	Chills
Hepatotoxicity	Thrombocytopenia	Fever
		Dizziness
		Depression
		Hair loss
		Anorexia
		Gastrointestinal ulceration
		Agranulocytosis
		Pneumonitis
		Toxic epidermal necrolysis
		Diffuse fibrosis

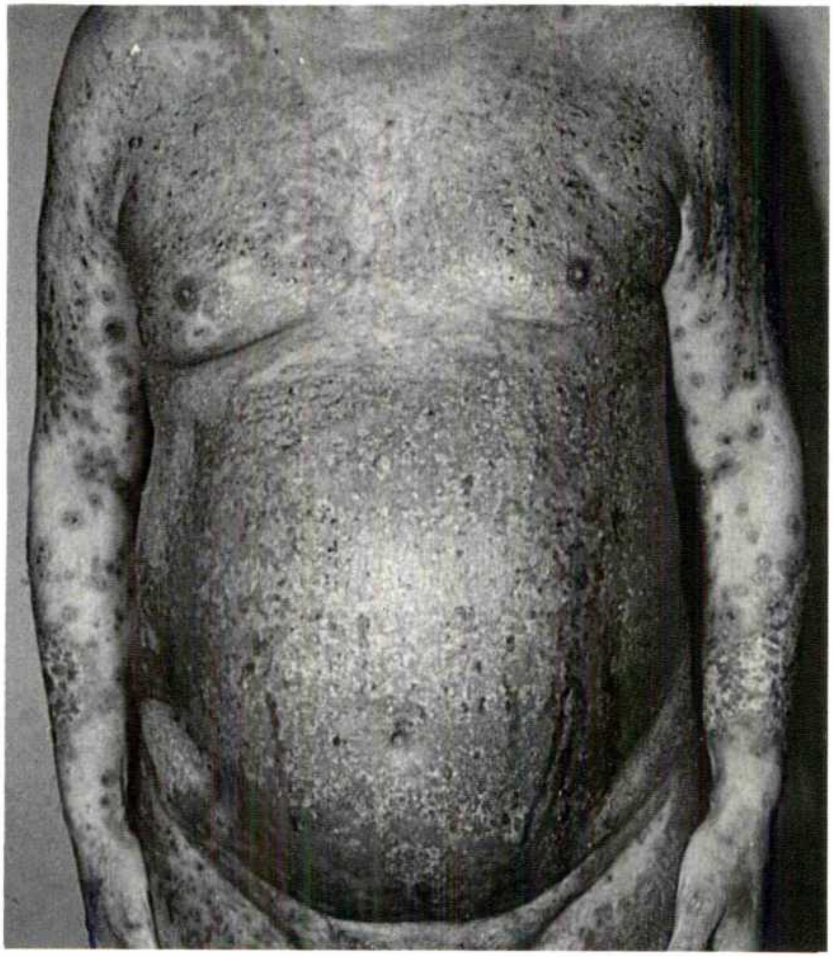

FIGURE 1. Toxic epidermal necrolysis with erosions within the psoriatic lesions has resulted from interaction between methotrexate and aspirin.

agranulocytosis is an extremely rare occurrence. All haematopoietic side-effects may arise especially following overdosing with MTX, which may lead to either a decreased renal function or interaction with other drugs. Good control of leukocyte and thrombocyte counts in particular is mandatory during MTX treatment.

SKIN AND MUCOSAL COMPLAINTS

Oral lesions of stomatitis are to be considered a warning sign of an overdose as is a burning sensation of the skin, and hair loss is rare. The severe reaction of a toxic epidermal necrolysis is associated with overdosages complicated by interactions (Fig. 1). Photosensitivity is not uncommon following MTX in high-dose regimes but, in general, this is not a problem with treatment of psoriasis.

HEPATOTOXICITY

The main reason for the current loss of MTX popularity as chemotherapy is without doubt the 'liver scare' and the recommendation of performing liver biopsies to monitor therapy,[3] a procedure which many dermatologists are hesitant to perform. Although it is well established that long-term MTX may induce liver damage which, in some patients, leads to fibrosis or cirrhosis,[4-7] more recent studies[8] have documented that such a cirrhosis is not aggressive. This is based on serial biopsies taken from 25 patients with MTX-induced liver cirrhosis 1–13 years after the cirrhosis was established (Table 3). Of 531 patients receiving MTX who have been followed-up since 1965, only one has developed liver failure. This was a patient who had continued to take the drug while not under the controlled conditions of the clinic, but elsewhere without appropriate monitoring.

The biochemical basis for the liver toxicity with MTX is not known. An action on DNA is unlikely, and folate depletion has been suggested to be an important factor. In addition to an increasing cumulative dosage, other factors associated with long-term liver toxicity in patients receiving MTX are alcohol intake, the combination of diabetes and obesity, and previous intake of arsenical and other hepatotoxic agents.

Liver control consists of initially a weekly and, later, a monthly check-up of liver transaminases with half-yearly estimation of the alkaline phosphatases. Until recently, our department has advocated serial liver biopsies at intervals of 1–1·5 years, depending on the findings of the preceding biopsy, and taking a liver biopsy when the total cumulative dose of MTX reaches 1·5 g. However, a new non-invasive test, using serum analysis of aminoterminal propeptide of type III procollagen (PIIINP),[9] allows a reduction in the number of liver biopsies required. According to this test, psoriatics with liver fibrosis and/or cirrhosis have increased values. It is still to be recommended that psoriatics receiving long-term MTX have a liver biopsy performed at least after an accumulated MTX dosage of 1·5 g but, if no significant fibrosis has appeared and the PIIINP remains normal, the liver biopsies may be reduced to a minimum as there is then apparently no great risk of the development of liver fibrosis or cirrhosis. Other non-invasive tests, such as liver scans using technetium 99 sulphur colloid and ultrasound or magnetic resonance imaging, are insufficient for detecting MTX-induced liver fibrosis.

PULMONARY COMPLICATIONS

Methotrexate may cause a pneumonitis, but this is rare in the treatment of psoriasis. The pneumonitis resolves rapidly after discontinuation of the drug and treatment with glucocorticoids.[10]

NEOPLASIAS

Because MTX is immunosuppressive, the drug theoretically carries the risk of carcinogenesis. There is, however, no sound evidence that MTX is to be considered carcinogenic in psoriasis. Retrospective as well as prospective studies in the U.S.A. have shown no increase in the rate of cancer in MTX-treated patients.[11]

MUTAGENICITY

Methotrexate gives negative results in the Ames test for mutagenicity.[12] Greater differences have been found in chromosomal studies[13] between psoriatics and normal controls than

TABLE 3. Results of serial biopsies in patients with cirrhosis

		Cumulative MTX dosage (mg)		Treatment (years)		OAC (years)		Cumulative cirrhosis index (grading of consecutive biopsies)											
Patient number	Sex/age*	Before cirrhosis	After cirrhosis	Before cirrhosis	After cirrhosis	Total	with histology	1	2	3	4	5	6	7	8	9	10	11	12
1 a	M/61	810	3,580	1	8	10	13	6	7	7	9	8	x	x	x	x	x	x	x
2 a	M/57	1,270	505	2	1	13	13	7	7	11	10	8	x	x	x	x	x	x	—
3 a,o	F/40	3,250	975	4	2	9	10	10	7	7	8	10	10	11	—	—	—	—	—
4 n	M/69	3,685	2,245	9	7	7	12	7	9	7	7	9	7	x	—	—	—	—	—
5 al	M/67	590	2,230	2	7	7	13	8	6	x	x	7	8	12	—	—	—	—	—
6 o	M/64	3,080	2,800	3	6	10	10	11	10	9	8	x	8	x	—	—	—	—	—
7 o	F/22	3,710	6,550	3	9	10	12	9	7	7	6	x	x	x	—	—	—	—	—
8 a, al	F/63	1,465	700	2	2	8	8	10	10	8	x	x	8	—	—	—	—	—	—
9 a	M/53	8,105	5,500	8	6	7	9	6	6	6	x	x	x	—	—	—	—	—	—
10	M/57	3,290	2,695	5	5	5	9	7	7	x	8	7	—	—	—	—	—	—	—
11	M/65	2,145	885	3	3	5	9	10	11	14	11	—	—	—	—	—	—	—	—
12	M/69	1,720	3,150	4	6	8	10	6	8	x	—	—	—	—	—	—	—	—	—
13 al	M/65	1,680	350	2	1	3	11	8	10	8	—	—	—	—	—	—	—	—	—
14 a, al	M/67	1,000	0	1	0	3	8	9	6	9	—	—	—	—	—	—	—	—	—
15 a, al	M/51	1,710	3,360	2	3	4	7	8	9	10	—	—	—	—	—	—	—	—	—
16	M/8	1,025	3,020	3	4	8	9	6	x	x	—	—	—	—	—	—	—	—	—
17	M/56	1,120	0	1	0	2	13	7	x	6	—	—	—	—	—	—	—	—	—
18	F/76	1,680	700	2	3	2	7	8	x	—	—	—	—	—	—	—	—	—	—
19 a	F/51	5,600	580	8	1	1	4	13	8	—	—	—	—	—	—	—	—	—	—
20 a, al	F/75	1,900	180	3	1	1	3	7	x	—	—	—	—	—	—	—	—	—	—
21	M/65	6,720	1,680	8	2	2	2	8	x	—	—	—	—	—	—	—	—	—	—
22	F/59	3,000	630	5	2	2	2	10	10	—	—	—	—	—	—	—	—	—	—
23 a	M/64	8,000	0	8	0	4	9	8	x	—	—	—	—	—	—	—	—	—	—
24	F/52	9,980	420	10	1	1	2	9	10	—	—	—	—	—	—	—	—	—	—
25 a	M/64	1,745	0	3	0	1	9	6	x	—	—	—	—	—	—	—	—	—	—

Zachariae and Søgaard.[8]

The findings are cumulative figures representing total scores for fibrosis, judgements of membrana limitans, fibrous destruction and regeneration. Each finding was graded from 1 to 4, Grade 1 being no pathological change; x indicates that no cirrhosis was found.

OAC = observation after cirrhosis; a = previously treated with arsenics elsewhere (and probably also vitamin A); al = previously heavy alcohol intake; o = obese; n = nephropathy with lowered renal function.

Patients 3, 4, 20 and 25 died without signs of liver failure.

*Age at time of cirrhosis diagnosis.

TABLE 4. Laboratory examinations before and after methotrexate treatment of psoriasis

Before therapy	During therapy
Haemoglobin, leukocyte and differential count, thrombocyte count	Leukocyte and thrombocyte counts, liver transaminases
Urine analyses, serum creatinine, creatinine clearance	Haemoglobin, PIIINP
Liver transaminases, alkaline phosphatases, bilirubin, PIIINP	Serum creatinine, creatinine clearance (optional)
Chest radiography	Chest radiography
	Liver biopsy, at least after 1·5 g accumulated methotrexate

PIIINP = aminoterminal propeptide of type III procollagen.

between psoriatics receiving MTX and those not receiving systemic treatment. So far, no malformations have been observed in the children of fathers receiving MTX near the time of the child's conception. This is in contrast to the children whose mothers were taking MTX during the first trimester of pregnancy. It is, therefore, to be recommended that MTX be avoided in both males and females who are planning to have children in the near future, and contraceptive measures should be continued for 3 months after the termination of therapy.

RENAL COMPLICATIONS

So far, with the dosages used in the treatment of psoriasis, renal impairment has not been reported. Kidney biopsies from 16 psoriatics treated for 1 month to 16 years[14] have been studied, using light and electron microscopy. Three patients have been treated for more than 10 years. Fourteen of the 16 biopsies were normal, and the minor pathological changes found in two biopsies were not considered to be MTX-related.

INTERACTION

Methotrexate interaction can occur with a number of other drugs. However, in practice, the only serious problems arise with the concomitant use of sulphonamides, especially if combined with trimethoprim, and salicylates (Fig. 1). Interaction with these drugs is due to competition for active renal tubular secretion and drug displacement. In addition, phenylbutazone and oxyphenbutazone are best avoided. Although a number of patients have benefited from the combination of MTX and retinoids, a warning may be appropriate. In our department, two out of 10 patients being treated with MTX plus etretinate developed life-threatening drug-induced hepatitis, a condition which has not been seen in any of the 531 patients taking MTX as monotherapy or in any of the 110 patients receiving etretinate on its own.

MODULATION

If leucovorin is administered a few hours following MTX, the acute side-effects are minimized. In cases of toxicity due to overdosing, 20 mg of leucovorin should be delivered parenterally with subsequent dosages every 6 hours, either orally or parenterally, according to needs.

GENERAL CONSIDERATIONS

The revised guidelines for MTX therapy in psoriasis[3] are recommendations which, in general, should be followed. It is mandatory that patients be given clear instructions, and that they receive special warning of drug interactions. The crucial factors in MTX treatment are that: indications should be good; the drug should only be used for severe and disabling psoriasis or active pronounced psoriatic arthritis; both the patient and the physician should be aware of the risk of side-effects and willing to undertake the necessary control (Table 4). If these rules are adhered to, the risk:benefit ratio for the drug is favourable for MTX as a valuable agent in psoriatic therapy.[3]

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Risk:benefit ratio in the treatment of psoriasis with systemic retinoids

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SUMMARY

This is a review of the efficacy of etretinate/acitretin in the treatment of psoriasis and of the currently reported side-effects. The data indicate that retinoids bring significant improvement (if not total clearing) with frequent low-morbidity but rarely serious side-effects. The most serious side-effect of etretinate/acitretin is teratogenicity.

Nearly a decade has elapsed since the introduction of synthetic retinoids for the treatment of skin diseases such as acne, psoriasis, congenital disorders of keratinization and neoplastic lesions of the epidermis.[1] This group of compounds has a toxicity that limits their clinical use. This is an evaluation of the risk:benefit ratio in the treatment of psoriasis with systemic retinoids.

BENEFITS OF RETINOID THERAPY FOR PSORIASIS

Among the synthetic retinoids that have been employed, two second-generation monoaromatic compounds, etretinate and acitretin, are efficacious in the treatment of psoriasis.[2] Isotretinoin has less of an antipsoriatic effect.[3] Most of the data evaluating the efficacy of these compounds in psoriasis are based on etretinate, which has been available in some countries for nearly 10 years.[4-9] However, the premarketing data indicate that acitretin, the free acid metabolite of etretinate, has a similar efficacy in psoriasis (Table 1).[10-15]

Total clearing

Total clearing of chronic plaque psoriasis (approximately 30% after 3 months with 0·6–1 mg/kg/day) may be observed after monotherapy, although this is not necessarily the rule. The decrease in the Psoriasis Area and Severity Index (PASI) score is approximately 60–70%, depending on the dosage.[10] Total clearing usually requires a combination of treatment [topical steroids,[16] anthralin (dithranol)[17] or PUVA[18-20]]. Acitretin is particularly effective for chronic

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TABLE 1. Summary of the efficacy of etretinate/acitretin in psoriasis

Type of psoriasis	Total clearing achieved		Rebound after stopping	Prolongs disease-free interval	Maintenance therapy avoids relapses
	Monotherapy	Combined			
Chronic plaque	30%	Yes, *PUVA	No	Possible*	Possible*
Pustular					
Generalized	Usually		No	Possible*	Possible*
Localized	Usually		No	Possible*	Possible*
Erythrodermic	Usually		No	Possible*	Possible*

*See text.

plaque psoriasis in combination with PUVA and significantly decreases the amount of UVA necessary for total clearing.[21] In some patients with disabling areas of psoriatic involvement, such as on the hands and feet, monotherapy usually produces a significant improvement. Both localized and generalized pustular psoriasis as well as erythrodermic psoriasis clear more frequently with etretinate/acitretin monotherapy.[7,22-25]

Rebound of psoriasis
Rebound does not usually occur after stopping acitretin/etretinate treatment.[26]

Disease-free interval
Little significant data are available on this aspect of treatment.

Maintenance therapy and relapses
Individual reports and observations indicate that some patients benefit from low daily doses (20 mg/day) and that dose tapering is followed by exacerbation. In a long-term double-blind study with etretinate (0·5 mg/kg/day), it was shown that, although relapses were sooner in patients receiving placebo, approximately 40% of those taking etretinate also relapsed during maintenance therapy.[26]

Efficacy in psoriatic arthritis[4]
Etretinate is effective in some cases of psoriatic arthritis and in Reiter's disease. Arotinoid ethyl ester (Ro 13-6298) has been successful in the treatment of severe psoriatic arthropathy previously unresponsive to non-steroidal anti-inflammatory drugs.

RISKS OF RETINOID THERAPY FOR PSORIASIS

The adverse effects of monoaromatic retinoids in the treatment of psoriasis can be divided into two groups: Type I (predictable) and Type II (unpredictable) (Table 2).[27]

Mucocutaneous side-effects
The frequency and intensity of mucocutaneous side-effects vary considerably, but rarely require discontinuation of treatment, and all are dose-dependent (Table 3).

Xerosis of the skin. This is the most common cutaneous side-effect and is frequently associated with pruritus and sometimes with erythema. The skin appears smooth and shiny.[4,28,29]

TABLE 2. Types of side-effects of monoaromatic retinoids

Type I	Type II
Pharmacological effect undesirable (same mechanism of action as therapeutic effect)	Toxic effect (involving organs or tissues in which no therapeutic effect is required)
Predictable	Unpredictable
Constant in all patients (therapeutic dose)	Rare (therapeutic dose)
	Individual susceptibility (idiosyncratic)
Early appearance (few days)	Late appearance (few weeks)
Intensity is function of daily dose	Intensity is function of cumulative dose
Rapidly reversible after discontinuation	Slowly reversible after discontinuation with possible after-effects

From J.M.Geiger, unpublished data.

Skin fragility. This may account for the blisters and erosions seen with etretinate.[30,31] Desquamation, particularly of the palms and soles, is frequent,[4] and facial dermatitis mostly on the central parts of the face can be seen. A dermatitis may develop in other sites, such as the trunk and legs, with occasional small haemorrhagic fissures.[32] Patients also complain of a 'sticky' sensation of the skin[33,34] while feeling cold and chilly.

Dryness of the mucous membranes. Cheilitis is seen in the majority of patients and may be useful for assessing dose and compliance. It occurs with a daily dose of $\geqslant 0{\cdot}3$ mg/kg etretinate and peaks at 6–8 weeks. The condition persists throughout treatment and improves rapidly on

TABLE 3. Mucocutaneous side-effects with etretinate/acitretin

Xerosis of skin; pruritus; erythema[28,29]
Skin hyperfragility: Palmoplantar desquamation; stickiness; facial dermatitis[31-34]
Dryness of mucous membranes (oral, nasal, vaginal and rectal); cheilitis; epistaxis[35]
Retinoid dermatitis[36-38]
Exuberant granulation tissue[42-44]
Photosensitivity[51-53]
Hair side-effects: hair loss; curly and twisted hair (pili torti)[54-57]
Nail side-effects: Paronychia-like reaction; nail-plate abnormalities with thinning, hyperfragility and softening[58-62]
Other side-effects:
    Nodular prurigo-like eruption[64]
    Leucocytoclastic vasculitis[67]
    Generalized oedema
    Erythema multiforme-like skin rash[161]
    Papular and pustular eruption of palms and soles[162,163]
    Ruptured striae atrophicae[164]

discontinuation of the drug. Scaling fissured inflammation of the lips has been reported, and other mucous-membrane sites, including the nose, rectum, urethra and vagina, may be affected.[35] Epistaxis is caused by thinning and fragility of the nasal mucosa.[29]

Retinoid dermatitis. This affects up to 50% of patients.[4] The erythematous papular rash may be difficult to differentiate from the underlying psoriasis,[32] mycosis fungoides[36] or eczema craquelé.[37] The extreme erythema, dryness and desquamation of the skin may lead to an erythroderma. Some workers have linked this erythroderma to low serum albumin levels which lead to the amount of free drug increasing to levels of toxicity.[38]

Whether these adverse effects on epidermal function are drug-related is not ascertained. Retinoid therapy decreases the number of tonofilaments and desmosomal attachments in the epidermis,[39] and leads to the accumulation of amorphous material both within and between keratinocytes.[40] Imperfect barrier function because of decreased epidermal and dermal cohesion is responsible for the xerosis and skin fragility.[41]

Exuberant granulation tissue. This affects the toe webs, nail sulcus and groin[4,42,43] and multiple pyogenic granulomas have also been reported with etretinate therapy.[44] This side-effect may represent an exaggerated healing response due to the retinoids[45] as, *in vitro*, dermal fibroblasts are stimulated and collagenase and gelatinase activity inhibited.[46] The presence of large amounts of acid mucopolysaccharides in the granulation tissue stroma may be related to the effects of retinoids on the synthesis of glycoproteins and acid mucopolysaccharides.[47,48]

Pyogenic granulomas may be the result of either the fragility of the skin allowing penetration of microorganisms or the inhibition of neutrophil migration to the tissues.[49]

Photosensitivity. Abnormal cutaneous photosensitivity is a frequent adverse effect of etretinate, affecting 7–9% of patients.[50] This low incidence of drug-induced phototoxicity suggests an idiosyncratic variability in the metabolism and/or pharmacokinetics of the drug.[51]

In vitro photohaemolysis, which screens the phototoxic potential of the drugs in the bloodstream, revealed that acitretin was phototoxic while etretinate was not.[52] Whether photosensitivity is due to thinning of the stratum corneum remains uncertain.[53]

Changes of the hair. More than 75% of patients report diffuse hair loss, but alopecia occurs in only approximately 20% and in association with high dosages. It is reversible on stopping the drug or reduction of the daily dose.[4,54] The hair may also become kinked, curly or twisted (pili torti).[55-57] Thinning of the hair is probably the result of telogen effluvium, and the condition and appearance of the hair may change on regrowth.[34]

Changes of the nails. The most common changes of the nails with etretinate are thinning, increased fragility, softening, loosening and eventual shedding.[58-62] Paronychia-like changes with redness and swelling of the skin fold around the dystrophic nail have been reported.[34] Granulation tissue in the nail sulci may develop independently of the dystrophy.[42] Nail growth rates increase during etretinate treatment.[63]

Other changes. Nodular prurigo-like eruptions[64] may be the result of acanthosis due to retinoids,[65] and a pityriasis rosea-like eruption has been confirmed by rechallenging with the drug.[66] Leucocytoclastic vasculitis was seen in five patients receiving etretinate and was considered to be drug-related.[67]

TABLE 4. Extracutaneous side-effects with etretinate/acitretin

Skeletal	Adults: Vertebral abnormalities DISH-like changes Osteophytes on vertebral bodies Spinal hyperostosis Calcification of anterior spinal ligament Extraspinal involvement Tendon and ligament calcification Osseous hyperostosis Children: Osteoporosis, skeletal pain Periosteal thickening Premature epiphyseal closure Slender long bones, narrow discs
Hepatic	Elevated liver enzymes in serum Severe hepatotoxic damage
Lipid	Hypertriglyceridaemia Hypercholesterolaemia
Ocular	Blepharitis Conjunctivitis Night-blindness
Neurological	Headache Intracranial hypertension
Muscular	Myalgias Muscle weakness
Aural	Otitis externa Earache
Genital	Sexual impotence (in males) Sexual dysfunction and menstrual changes (in females)
Teratogenic	Spontaneous abortion Limb, craniofacial and CNS malformations
Biological	Elevated creatinine kinase Decrease of erythrocytes Increase of monocytes
Miscellaneous	Fatigue Nausea Renal oedema Lymphoma Gynaecomastia

Extracutaneous side-effects

These are summarized in Table 4.

Skeletal toxicity in adults. Chronic retinoid toxicity of the bones has been reported in adults taking etretinate. Skeletal changes, including demineralization, rarefaction of bone, cortical hyperostosis, periosteal calcification, thinning of the long bones or premature epiphyseal closures, are well-known symptoms of chronic hypervitaminosis A syndrome.[68] The most commonly involved areas are the vertebrae and extraspinal ligaments.[69]

These changes occur more often in the cervical than in the thoracic or lumbar spine.[70,71] The abnormalities are related to ageing and the diffuse idiopathic skeletal hyperostosis (DISH)

TABLE 5. Skeletal abnormalities reported in children taking etretinate

Authors (ref.)	No. of cases (total)	Age (years)	Gender	Dose (mg/kg/day)	Duration (years)	Skeletal abnormalities
Halkier *et al.*[88]	1	11·4	F	0·78	7·4	Periosteal thickening of ulnar and metacarpal bones Progression of periosteal thickening of fibula
	1 (2)	7·4	F	0·86	0·5	Mild disc narrowing
Glover *et al.*[89]	0 (1)	8·5	M	0·5	1	No bony abnormalities detected using ^{99m}TcMDP
Van der Schroeff, Van der Rhee[90]	0 (19)	4-14 (mean 8·9)	8 M 11 F	0·3 (mean 0·6)	8–66 mos (mean 33)	No skeletal toxicity
Rosinska *et al.*[92]	1 (10)	3-15 (mean 9·1)	7 M 3 F	0·2-1	—	Osteoporosis

^{99m}TcMDP = technetium 99m methylene diphosphonate.

syndrome.[72-76] The radiological and pathological features of this syndrome, according to Resnick,[77] are: 'flowing' calcification and ossification of the anterolateral aspects of at least four adjacent vertebral bodies; preservation of the intervertebral disc space in the involved areas and absence of radiological changes of 'degenerative' disc disease; absence of apophyseal bony ankylosis and sacroiliac erosion. Spinal hyperostosis is the result of bony accretion on the anterosuperior and anteroinferior margins of the vertebral bodies.[78-82] Calcification of the anterior spinal ligament and osteophytes on the vertebral bodies have also been reported.[83]

Tendon and ligament calcification[84,85] was bilateral in 84% of cases and multifocal in 69%,[74] and affected, in a decreasing order of frequency, the ankle, pelvis, knee, shoulder and elbow.[4] Hyperostosis of the calcanei at the insertion of the plantar ligament and hyperostoses of the anterolateral lips of the acetabula were also found.[83] Evaluation of the biochemical and hormonal parameters of bone metabolism (serum calcium, alkaline phosphatase, osteocalcin, calcitonin and parathormone levels) revealed no significant changes after 6 months of acitretin treatment (0·4–0·8 mg/kg/day).[86]

The incidence of skeletal toxicity in adults is probably underestimated. In one study, 84% of 38 patients showed extraspinal tendon and ligament calcification on radiography, and 29% showed spinal involvement similar to diffuse idiopathic hyperostosis after 5 years of etretinate therapy.[74] Scintigraphy of the bone in patients treated with etretinate revealed no changes.[87] The severity, but not the incidence, of these abnormalities appears to be related to both the dose and duration of treatment.[34]

Skeletal toxicity in children. In children receiving etretinate for psoriasis between 1980 and 1989,[88-92] skeletal alterations were less frequent than expected (Table 5). Only three out of 32 children had skeletal abnormalities, which consisted of osteoporosis, periosteal thickening, premature epiphyseal closure, slender long bones, ossification of the interosseous membranes of the forearm, skeletal pain, disc narrowing and cortical bone resorption. These changes were usually asymptomatic and independent of changes in serum calcium, phosphate and alkaline phosphatase concentrations,[91] and were probably related to individual susceptibility, treatment for 5 years of more and high doses of etretinate.

Vitamin A is essential for normal bone metabolism[93] and, in experimental animals, bone growth, including appositional bone formation, is accelerated by excessive doses of vitamin A.[94] Osteoblasts synthesize osteoid which, with optimal blood calcium and phosphate concentrations, mineralizes to form bone.[69] As retinoids increase epidermal interleukin-1 (IL-1),[95] it is likely that retinoids also enhance IL-1 production in bone. Granulocyte/macrophage colony stimulating factor may be important in the relationship between osteoblasts and osteoclasts.[96] The toxicity is the result of the acceleration of bone and cartilage resorption.[85]

Liver function abnormalities. Approximately 20–30% of all patients receiving etretinate develop temporary abnormal liver function tests,[4] indicated by a slight transient increase in aspartate transaminase (ASAT, SGOT), alanine transaminase (ALAT, SGTP), lactate dehydrogenase (LDH), gamma-glutamic transpeptidase (gamma-GT) and alkaline phosphatases. These usually occur after 0·5–2 months of treatment,[34] but are completely reversible after either reduction of the dose or discontinuation of the therapy.[97]

Twenty-four reported cases of severe hepatotoxic damage (Table 6), confirmed by liver biopsy, can be divided into three categories. The first was hepatitis[98-102] that occurred only in women aged 40–70 (mean 61) years except in one (male) case.[103] The condition developed after 2–18 weeks and appears to be idiosyncratic, as suggested by exclusion of other possible aetiological factors and near resolution of the biochemical abnormalities after discontinuation of the drug. In two patients, acute hepatitis resulted from a probable drug hypersensitivity, suggested by its association with fever and eosinophilia.[104,105] Four cases of chronic active hepatitis were also reported,[106,107] comprising a second type of hepatotoxicity. There was one case of mild cirrhosis after combination therapy with methotrexate and etretinate.[108]

Several studies including liver biopsies to identify possible chronic hepatotoxicity in 79 patients receiving long-term etretinate treatment (up to 6 years)[109-113] showed no hepatotoxicity. A prospective study including liver biopsies from 17 patients who had received etretinate treatment for 3 years also revealed no significant liver damage. Recently, Camuto[114] evaluated liver function and morphology in 18 patients treated with etretinate for at least 5 years. Six patients showed fatty infiltrations and two showed mild fibrosis. One patient with fibrosis and another with cirrhosis had both received previous treatment with methotrexate.

Hepatotoxic reactions are well-known complications of long-term high-dose retinol therapy.[115] Etretinate has a much lower affinity for the liver than does retinol. The drugs differ in their aromatic ring and ester end-group.[104] Most of the synthetic retinoid-induced hepatotoxic reactions are due to etretinate,[116] perhaps because the tissue:blood concentration of the drug in the liver is the highest (approximately 30-fold) and the drug is stored in the liver, suggested by the fatty infiltration seen on biopsy after prolonged treatment.[117-119] The main site of etretinate storage is body fat and Ito liver cells are probably the storage sites of lipids and retinyl esters.[120] In liver biopsies of patients receiving etretinate for at least 5 years, Camuto[114] found a higher lipid level in their Ito cells than in those of controls.

Serum lipid alterations. Hyperlipidaemia is one of the most common side-effects of retinoid therapy.[34] Plasma cholesterol and triglyceride values increased in patients receiving monoaromatic retinoids.[121] In approximately 50%, serum triglyceride values rise to more than 2·5 g/l and, in approximately 30–40% of patients, the serum cholesterol exceeds 3 g/l.[122-124] The

TABLE 6. Summary of literature on etretinate hepatotoxicity

Authors (ref.)	No. of cases	Age	Gender	Dose	Duration of treatment	Liver function abnormalities	Liver biopsy findings
Fredriksson[106]	2	?	F	—	> 6 months	Elevated B,ALT,AST	Chronic active hepatitis
Thune & Mork[98]	1	54	F	50 mg/day	4 months	Elevated B,ALT,AST,AP	Centrolobular necrosis
Van Voorst *et al.*[99]	1	70	F	1 mg/kg/day	4 months	Elevated ALT,AST	Portal hepatitis
Van Voorst *et al.*[99]	1	61	F	1 mg/kg/day	3 months	Elevated ALT,AST	Centrolobular necrosis
Weiss *et al.*[107]	1	39	M	0·6 mg/kg/day	6 months	Elevated ALT,AST	(No liver biopsy)
Weiss *et al.*[104]	1	74	F	0·75 mg/kg/day	1 month	Elevated ALT,AST,AP,EO	Chronic active hepatitis
Vahlquist *et al.*[100]	1	64	F	0·85 mg/kg/day	3 months	Elevated ALT,AST,AP	Hepatitis, liver cell necrosis
Foged & Jacobson[101]	1	49	F	50 mg/day	5 weeks	Elevated ALT,AST	Lobular and portal hepatitis
Foged & Jacobson[101]	1	63	F	50 mg/day	2 weeks	Elevated ALT,AST	Confluent bridging, piecemeal necrosis
Gavish *et al.*[102]	1	58	M	1 mg/kg/day	3 months	Elevated B,AP	Canalicular cholestasis, periportal hepatitis
Khouri *et al.*[105]	1	73	M	50 mg/day	1 month	Elevated B,ALT,AST,AP,EO	Lobular, portal, periportal hepatitis
Beck & Fosed[108]	1	47	F	1 mg/kg/day	4 months	Elevated B,ALT,AST,AP	Mild cirrhosis
Grimaud *et al.*[103]	1	62	M	50 mg/day	3 months	Elevated ALT,AST,AP	Eosinophilic necrosis, microvascuolar steatosis
Total	14						

ALT = alanine aminotransferase; AST = aspartate aminotransferase; AP = alkaline phosphatase; EO = eosinophilia; B = bilirubin.

changes are manifest after 2 weeks of treatment and usually peak by 8 weeks.[125,126] The increase in triglyceride level was dose-related some studies,[123] but not in others.[125]

Reducing the dose of etretinate may reduce the lipaemia and, 4 weeks after stopping treatment, values will return to normal, indicating that the prolonged elimination half-life of etretinate does not delay reversal of the hyperlipidaemia.[123,127] The increase in serum lipids is not related to the pretreatment levels of triglycerides and cholesterol.[125,126] Patients with predisposing factors, such as obesity, alcoholism, diabetes mellitus, familial hyperlipidaemia and nicotine abuse, have a higher incidence of hyperlipidaemia with etretinate.[121]

Fractionation studies of triglycerides show that the largest increase is in the very low-density lipoprotein (VLDL) fraction, with smaller increases in low-density lipoprotein (LDL) and no change in high-density lipoproteins (HDLs).[121,124-126] The largest increase in cholesterol is in the LDL, with a smaller increase in the VLDL and a small decrease in HDL,[124,126,128] presumably a reflection of changes in HDL composition rather than a decrease in the number of HDL particles. Lipoprotein structure is dependent on its apoprotein constituents, which form an interface between the non-polar lipid core (triglyceride and cholesteryl esters) and the outer layer (phospholipid and free cholesterol). After etretinate therapy, increased concentrations of apoprotein B, the major component of VLDL and the only apoprotein in LDL, have been reported. A small increase in total apoprotein A, the major constituent in HDL, was also detected.[124] Levels of apoprotein A1 and A2 remained unchanged. It may be that chronic use of oral retinoids leads to an increased risk of atherosclerotic cardiovascular disease.[121]

Although the mechanisms of retinoid hyperlipidaemia are not known, according to Marsden[121] there are four possibilities: (1) Increased absorption of dietary lipids; (2) Increased chylomicron production or reduction of its clearance; (3) Increased synthesis of triglycerides and cholesterol in the liver; (4) Increased synthesis or decreased catabolism of VLDL and LDL.

Ocular manifestations. Etretinate has side-effects involving the eyelid, conjunctiva, cornea and retina. Blepharitis may occur due to an increased susceptibility of the eyelid to sunburn.[29] Blepharoconjunctivitis is the most frequent ocular adverse effect.[129] The symptomatology is variable, involving dryness with slight irritation or pain. Dryness of the conjunctiva prevents the wearing of contact lenses.

A recent report[130] on four patients receiving etretinate therapy demonstrated a significant colour blindness in the blue–yellow spectrum and extending to red–green with higher cumulative doses. Deuteranomalous indications were revealed by anomaloscopy. Another retinal dysfunction was the loss of dark adaptation, confirmed by electroretinography (ERG) which records changes in the electrical potential in the retina after light stimulation.[131]

Vitamin A (retinol) is essential for vertebrate retinal photoreceptor function. The metabolism of retinol in the optic system allows the synthesis of the chromophore 11-*cis* retinal,[132] which combines with the protein opsin in the retinal pigment epithelium to form rhodopsin,[133] the visual protein of the photoreceptors. A recent study[134] showed that retinoic acids inhibit the ocular retinol dehydrogenases *in vitro*, which is followed by loss of 11-*cis* retinal to the photoreceptors which may lead to retinal dysfunction.[135] Differences in the binding affinities of particular retinoids to various opsins may explain the diversity of retinal side-effects. Some workers suggest that the eyes of patients receiving long-term etretinate should be examined regularly with special attention to colour vision and dark adaptation.[130]

Central nervous system effects. An increase in intracranial pressure was seen in three patients receiving etretinate therapy.[136,137] Intracranial hypertension was associated with headache, nausea, vomiting, impaired vision and papilloedema.

Muscular effects. Frequently, patients taking etretinate present with intermittent muscular pain. An increase in muscle tone in patients taking etretinate for 3 years or more[138] sometimes caused a slightly stooping posture and stiffness. The upper trunk, middle and lower parts of the thoracic and lumbar spine, and the leg muscles are usually involved. Histological and ultrastructural studies revealed muscle damage due to etretinate as a segmental muscle necrosis,[139] and electromyography has demonstrated subclinical muscle damage of short duration and low-amplitude action potential.[140]

Aural effects. Otitis externa and earaches have been reported[141,142] with etretinate treatment. The mechanisms of these adverse events may be related to the increases in apocrine sweat gland secretion, such as of the ceruminous glands, as shown in the external ear canal of dogs treated for more than 6 months with high doses of etretinate.[143]

Reproductive system effects. Investigation of male patients receiving etretinate for changes in reproductive capacity or pathological alterations in spermatograms has revealed no adverse effects.[144] Spermatozoal DNA showed no significant changes on impulse cytophotometry,[145] and fructolysis, the main source of energy in human spermatozoa, was apparently not affected by therapeutic doses of etretinate.[146] Several cases of impotence in men have been reported, but a relationship to the drug was not confirmed.[147] Sexual dysfunction and menstrual changes have been described in female patients.[148]

Teratogenicity. Vitamin A and its derivatives are well-known potent teratogens. In addition to their embryopathic effects, these medications also lead to spontaneous abortion in about one-third of pregnancies,[149] and etretinate-exposed pregnancies were associated with a relatively high risk of congenital malformations including craniofacial, limb and central nervous system defects.[150] Cardiac abnormalities have not been reported with etretinate, unlike the pattern of abnormalities seen with isotretinoin.[151] Of 37 pregnancies occurring within 2 years of stopping treatment, only three congenital malformations were considered possibly etretinate-related.[152] Acitretin is also a potent teratogen,[153] but there are no reports of embryopathies with this retinoid.

Biological abnormalities. No alteration of thyroid function during etretinate treatment has been reported,[154] although blood glucose values may decrease slightly.[155] An increase of blood monocytes and decrease of erythrocytes have been noted after long-term etretinate treatment, and creatine kinase was raised in two patients.[156]

Miscellaneous effects. Only three cases of oedema[157,158] and one case of gynaecomastia[159] have been reported and, although there were two cases of lymphoma, whether these were drug-related is not known.[160] Fatigue has been reported in 15% of patients, and gastrointestinal tolerance is generally good with only 2% complaining of nausea.[8]

CONCLUSIONS

The risk:benefit ratio of any therapy is difficult to evaluate as much depends on the specific morbidity of the disease in any given patient. After nearly 10 years of use, except for the teratogenicity, etretinate may be considered a relatively safe drug. Severe morbidity resulting from the retinoid-induced side-effects is rare, and most of the side-effects are reversible after stopping therapy. In evaluating the benefit of etretinate/acitretin for psoriasis, the morbidity of the benign mucocutaneous side-effects should be considered as they have an effect on the quality of life. Etretinate/acitretin as monotherapy does not achieve total clearing in all patients, but may be a significant adjuvant therapy for PUVA by reducing the carcinogenic risks of that modality. Only long-term evaluation of these parameters will determine the position of retinoids in comparison to CyA in the treatment of psoriasis.

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